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Edited by
C. E. A. Winslow

It is characteristic of Science and Progress that they continually open new fields for our vision.—Pasteur

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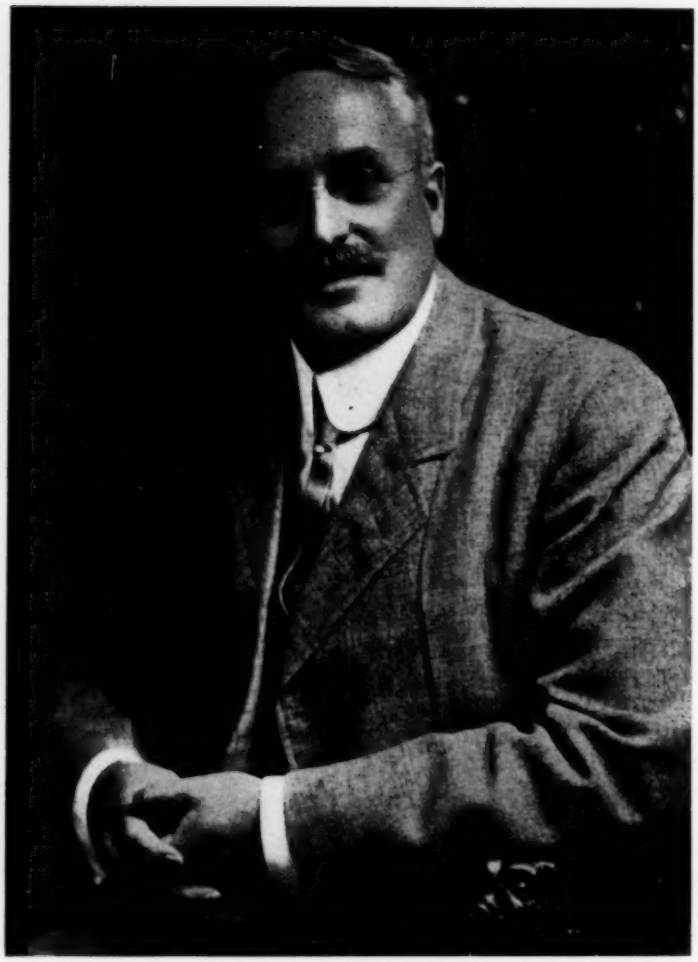

Frank Fairchild Wesbrook

FRANK FAIRCHILD WESBROOK

H. W. HILL

Frank Fairchild Wesbrook was born July 12, 1868, at Oakland, Ontario, Canada, and died, at the age of fifty, on October 20, 1918, at Vancouver, B. C., after an illness of a year's duration.

From the year 1913 and until the time of his death he was President of the University of British Columbia.

He leaves a widow, Mrs. Annie Wesbrook, and a daughter, Helen Wesbrook, the latter in the senior year of the Arts College.

President Wesbrook took his Bachelor's and Master's degrees at the University of Manitoba in 1887 and 1888, and the degrees of M.D. and C.M. in 1890 from that University and McGill College; while in later years the Universities of Manitoba, Toronto and Alberta conferred upon him the degree of LL.D. During 1891–1893, he served as Professor of Pathology at the University of Manitoba; thence he went to Cambridge University in 1893–1895 as John Lucas Walker student, where he engaged in research work in pathology and served as a University tutor under Gaskell.

In 1895 he was called to the directorship of the Department of Pathology, Bacteriology and Hygiene at the University of Minnesota, filling this position as professor throughout his connection of eighteen years with this University.

Dr. Wesbrook's chief work was in Bacteriology—particularly as related to public health. With O. McDaniel and L. B. Wilson he published the famous "Types of Diphtheria Bacilli" which did so much to simplify, and make accurate, diphtheria diagnosis. He was one of the earliest and most foresighted investigators and advocates of chlorine sterilization of water supplies, which has become an immensely important factor in public health.

He was Director of the Minnesota State Board of Health Laboratories until 1913 and for two periods was a member of the Minnesota State Board of Health.

In 1906 he was appointed to the Deanship of the Medical School of the University of Minnesota and efficiently administered his trust until he was invited to the Presidency of the University of British Columbia and resigned his position at Minnesota.

He threw himself ardently into the development of the new University at Vancouver and throughout the war was especially concerned in the training of young men and women for war service and for the coming duties of reconstruction.

Dr. Wesbrook was an active worker in the interests of public health and for many years has been recognized in the public health councils of this country and of Canada. He was Chairman of the Laboratory Section of the American Public Health Association in 1901, and was elected President of that Association in 1905, serving during the Boston meeting. He was a charter member of the American Society of Bacteriologists. In 1912 he was chosen President of the section on State and Municipal Hygiene of the International Congress of Hygiene and Demography and for ten years served as a member of the Advisory Board of the Hygienic Laboratory at Washington, D. C.

His powerful physique, splendid bearing and great cordiality made him an impressive and attractive figure everywhere.

His demise excited general regret, and no one who knew him but feels a great personal, as well as professional, loss; the world was richer for his presence and poorer for his departure.

AGGLUTINATION[1]

R. E. BUCHANAN

From the Bacteriological Laboratories, Iowa State College, Ames, Iowa

The address of a retiring president of a scientific society at this time might well be devoted to a discussion of the relationship of the science in question to the conduct and winning of the war. Certainly bacteriology has played a major rôle in this drama. Its contributions have been of fundamental importance. Therefore, notwithstanding the fact that there are many who have been in far closer contact with the war triumphs of our science than have I, it is probable I should have essayed the task, had it not been for the events of the past few weeks. The war, we confidently believe, has been won. May we not, then, address ourselves to the pursuits of peace instead?

I shall confine my remarks to an entirely different phase of our science, namely, the phenomenon of agglutination, particularly to certain physico-chemical conceptions and their bearing upon a theory of agglutination.

For many years the terminology and conceptions of Ehrlich as they related to the phenomenon of agglutination have held sway in our texts and have in general proved fairly adequate for an introductory or rather casual explanation of this phase of immunology. They have served as guides in our discussions of the differentiation and recognition of bacteria by specific sera, and of the diagnosis of disease by use of specific bacteria. In some quarters, perhaps as a natural result of our anti-Teutonic bent in the Great War, there are signs of revolt against the dominance of such expressions. Witness a recent statement by Moore (1917) in the British Medical Journal:

[1] Presidential Address delivered before the Society of American Bacteriologists at its Twentieth Annual meeting, December 27, 1918.

Progress in our knowledge of the biochemistry of immunity will be delayed so long as the modes of thought connoted by the series of *ad hoc* terms first introduced by Ehrlich and since reduplicated by others, are allowed to dominate the clear view that the laws of immunity are the laws of reaction by adsorption in colloidal solution. There is nothing new or ever was in the side chain theory except the fantastic terms. . . . . The *pioneer* work on immunity which has carried us so far forward in many a domain of pathology and bacteriology was that of Bordet, Gengou and others of the French School; it was burdened from Germany with the intolerable weight of a useless philosophy of jangling terms for a type of reaction well known in colloidal chemistry. . . . .

Further,

All the laws of immune reactions can be illustrated by colloidal systems which are not and never were living and the preservation of a special set of terms is redundant.

Also in the same vein the statement by Roberts (1917),

I wish to ask whether it is not time to simplify the theory of immunity. Much modern work seems intended to support "orthodox Ehrlichism," though by the multiplication of sub-hypotheses its confusion grows worse confounded. The terms employed in general physiology should thus be sufficient for bacteriology, and observers of fresh phenomena ought to be chary of coining new words. Their hasty multiplication usually implies some additional hypothesis. It is characteristic of a false explanation to require an increasing number of sub-hypotheses while a real one abolishes a multitude of superfluous terms and, displaying a phenomenon as the "functions of known variables, by such a disclosure is essentially a simplification."

These caustic criticisms to the contrary notwithstanding, it probably will be long before the Ehrlichean conception of receptors of the second order passes from our texts on bacteriology and immunity. However, the phenomenon of agglutination has been found to be complex, so complex that the older explanations and conceptions fit the facts only relatively and poorly. We hear now of co-agglutination and conglutination, the fixation of complement in agglutination, of acid or hydrogen ion agglutina-

tion and precipitation. When we expand and revamp the Ehrlichean terminology to fit the new facts we are in danger of being lost in a forest of verbiage. Physical, colloidal, and bio-chemistry have come to our rescue, and have contributed facts which will assist us in formulating a better and more consistent conception of the agglutination phenomenon. I have derived much pleasure from a review of some of this work and an attempt to correlate and systematize it into a well coördinated theory, drawing in part upon experimental results of my own for illustration. Other than this illustrative material there is little that is new in my discussion.

A. HISTORICAL

Investigators who followed the initial discovery of the phenomenon of bacterial agglutination by Gruber and by Widal may be divided into three groups; one, with Ehrlich as leader, studied the phenomenon of serum agglutination quite intensively, and built up around their observations a relatively complex portion of the side chain theory of immunity. Ehrlich, Eisenberg, Volk, Wasserman and others introduced a formidable terminology for the phenomena observed. Much of this terminology proved valuable from both theoretical and practical points of view, but there was relatively little effort to base the theories upon well recognized laws of colloid chemistry.

A second group of investigators interested themselves in efforts to work out methods of bacterial identification by agglutination produced by substances other than the homologous antisera. The first of these workers, apparently, was Malvoz (1897, 1899) who studied the flocculating effect of many chemicals, and concluded that the typhoid bacillus was specifically agglutinated by formaldehyde. Beco (1899) and others, however, showed this reaction to be non-specific. This type of investigation was also pursued by Bossart (1898) and by Sabrazes and Banzierés (1899), who found many substances which would cause bacterial agglutination, but none which made practicable the satisfactory differentiation of species. Michaelis (1911) concluded that

the typhoid bacillus in suitable suspension shows a specific agglutination in a definite hydrogen ion concentration in a suitably buffered solution. Beniasch, (1911) extended this observation to a large number of other bacteria, concluding that acid agglutination could be used as an aid in the recognition of many species. Favorable results were secured by many other investigators as Rost (1911), Jaffe (1912), Poppe (1912), Schidorsky and Rein (1912), Stepanoff (1912), Grote (1913), Sgalitzer (1913), and Gillespie (1914). Crendiropoulo and Amos (1904) showed that certain salts favored agglutination. Liefmann (1913) believed it possible to differentiate bacteria by a process of salting out similar to that used with proteins.

The third group of investigators have approached the subject from the standpoint of physical or colloidal chemistry, and have attempted to correlate results secured in bacterial agglutination with similar non-specific phenomena in chemistry. These writers do not use the Ehrlichean terminology, and their results and conclusions do not generally find place in our texts. It seems demonstrable however, that they have pursued the course which is leading to a logical and satisfactory explanation of the immunity phenomena. Among them Bordet (1896) clearly recognized and proved that in specific agglutination there are two distinct phases, first, the impression or sensitization of the bacterial cell by the agglutinins and second the precipitation or agglutination of the cells due to the action of salts. He emphasized the fact that this agglutination was homologous with the flocculation of colloidal solutions by salts. His results were extended and confirmed by Joos (1902). Two years later three important contributions were made, emphasizing the homology of the agglutination reactions with colloidal phenomena. Bechhold (1904) carried out a well planned series of tests with mastic and with bacteria both sensitized and unsensitized. His work is so conclusive and gives such excellent illustrative material for class and laboratory demonstration that it is surprising that so little use has been made of it. He investigated the activity of various ions, showed that bacterial cells carry electric charges, determined the agglutinating limits of many substances, and demonstrated both

regular and irregular series. Landsteiner and v. Jagic (1904) called attention to the analogy between the action of colloidal silicic acid and the immunologic reactions. Neisser and Friedmann (1904) duplicated much of the work of Bechhold and reached similar conclusions. Zangger (1905) also emphasized the complete agreement between the action of immune bodies and colloids. Landsteiner and Stankovic (1906) studied the adsorption of proteins and the binding of agglutinins. In the same year Porges (1906) carried on numerous experiments. In more recent years contributions have been made through the work of Millen (1909), Bordet and Streng (1909), Dreyer and Douglas (1910), Michaelis (1911), Beniasch (1911), Krumwiede and Pratt (1912), Michaelis and Davidsohn (1912), Ostwald (1912), Dean (1912), Chick and Martin (1912), Landsteiner (1913), Ostwald (1913), Schmidt (1913), Tullochs (1914), Walpole (1914), Hofmann (1914) and Priestley (1917). These workers have placed our knowledge of bacterial agglutination on a relatively sound basis.

B. WHY DO BACTERIA AGGLUTINATE?

Before undertaking a consideration of the question, why do bacteria agglutinate, it may be well to ask, why do bacteria *not* agglutinate? An observation of a hanging drop of bacteria will usually show either active motility or active Brownian movement. Cells constantly collide, or at least approach each other closely, then they move apart, apparently repulsing each other, and remain quite uniformly distributed over the field. The factors which may have to do with agglutination are the repulsion of the cells for each other, the vigor of their movements whereby they collide or enter each others sphere of effective influence, and the attraction which the cells may possess for each other. Agglutination occurs whenever the forces tending to draw these particles together are more powerful than those of repulsion.

Let us first consider the factors which determine the repulsion of bacterial cells for each other. It has long been known that particles suspended in water usually bear an electric charge.

When placed in an electric stream they generally move toward the anode, that is, they bear a negative charge; in a few cases the charge is reversed and they move to the cathode. Hardy (1899) showed that the particles of a colloidal suspension of boiled egg-white moved to the anode, but that the direction could be reversed by the presence of suitable electrolytes. This observation was extended to bacterial suspensions by Bechhold (1904) who found that bacteria whether heated or unheated wandered to the anode. This fact has been abundantly confirmed by other investigators since. Among these were Neisser and Friedmann (1904) Porges (1906), Porges and Prantschoff (1906), Michaelis (1909), Beniasch (1911), Schmidt (1913), Landsteiner (1913), Arkwright (1914), and Priestley (1917). Apparently suspensions of bacteria as ordinarily grown are negatively charged and the mutual repulsion of the cells may be due to these charges.

The occurrence of the phenomenon of flocculation or agglutination is in itself evidence that there must exist a force of attraction, under certain circumstances at least. This force is probably surface tension. If the bacterial cells do not repel each other, nor attract each other *per se*, the phenomenon of surface tension explains the fact that they remain together.

It is evident then, that we have at least a partially adequate explanation of why bacteria under certain conditions remain in suspension, and why under other conditions they may cling together. We may regard the similar electric charge as constituting the repulsing agency, and surface tension as the attracting agency. A study of the agglutination phenomenon then resolves itself into a consideration of the means whereby these two forces may be modified, increased or diminished. Agglutination occurs whenever the similar electric charges are decreased to amounts such that they will no longer overcome the pull of surface tension. Or conversely, surface tension may be increased until it overcomes the dispersion effect of the similar charges. What are the factors which govern the intensity of these forces?

C. CATAPHORESIS

First let us consider methods which may be used to modify or even reverse the charge upon bacterial cells. To Hardy (1900) we owe a knowledge of the fact that particles of denatured proteins may be deprived of their charge, that is, rendered isoelectric with the solution, or may have their charges reversed by the addition of electrolytes. Particularly marked was found to be the activity of acids, alkalies and salts. After suitable adjustments it was found that the particles moved neither to anode nor cathode, and the particles flocculated. The addition of further small quantities of either alkalies or acids caused the particles to disperse and acquire the property of traveling in the electric field. It is evident then that with certain suspensoids electrolytes will cause flocculation when the particles are made isoelectric. Is the same true of bacterial suspensions? Beniasch (1911) has shown that a change in hydrogen ion concentration to a point where typhoid bacteria agglutinate does not necessarily neutralize the charge on the bacterial cells, for when showing acid agglutination they may still pass to the anode. The apparent discrepancy possibly arises from the fact that bacteria do not consist of a single colloid and this agglutination may represent the isoelectric point of some one of the constituents. Arkwright (1914) indeed found that in *Bacillus typhosus* there are two distinct substances agglutinable by acids, and at least one of these flocculates at its isoelectric point. We may safely conclude that the charge carried by a bacterial cell, or at least by its various components, can be altered by additions of various substances to the solution.

It may be accepted then that the presence of electrolytes may modify the charge on a bacterial cell. But how? Apparently by the adsorption of ions or particles bearing an opposite charge. Conversely, the charge on the cell may be due to the various substances adsorbed, frequently to the ions. It is necessary that there be such an affinity between the positive cation, for example, and the negative bacterial cell that the latter will adsorb the former. The dispersive force is thus gradually neutralized.

D. DENATURATION OR SENSITIZATION

It should also be noted that the ability of a particle to adsorb an ion may be modified. Proteins in particular may be changed from a form difficult to flocculate in any electrolyte concentration to one flocculated by even slight concentration. Hardy (1899) pointed out that coagulation or precipitation of proteins as a result of hot water involved two quite distinct processes; first, a fundamental change in the protein which modified its characteristics markedly, and second the agglutination of these modified particles under the influence of electrolytes. This transformation of a protein he termed the process of denaturation. Apparently, then, it is possible to modify the adsorptive powers of particles for ions. When this adsorptive power is increased we may speak of the process as one of denaturation, or when dealing with bacteria, as sensitization.

Tentatively we may classify the methods of denaturation or sensitization under two heads; first, a change in the chemical constitution or atomic arrangement, such as that produced by heat in presence of water or the action of some chemical; second, a change due to adsorption of some substance either a colloid or an electrolytic ion which in turn modifies its adsorptive powers. Agglutination may be produced by sensitization or denaturation of bacteria so that they will adsorb substances which will so modify their electric charges that the dispersive forces are weaker than the attracting forces.

E. FACTORS INFLUENCING AGGLUTINATION

1. Nature and composition of antigen

The nature and composition of the antigen are important factors influencing agglutination. A suspension of bacteria as ordinarily prepared for agglutination tests is a very complex mixture. The bacterial cells themselves constitute a suspensoid, and each bacterial cell is in itself a colloid mixture. It is apparent that these various constituent colloids may behave in a sense independently, and that therefore, the bacteria may

show distinct zones of agglutination. Table I gives an example of this for meningococci. It will be noted that the particular antigen shows flocculation at PH values of 3.0 and 5.0. This phenomenon was apparently first shown for *B. typhosus*, by Arkwright (1914) who found one zone of specific agglutination,

TABLE 1

Effect of variation in concentration of antigen on agglutination by hydrogen ions. Suspension of washed meningococci in distilled water. Turbidity of suspensions given in silica standard equivalents. 56° for thirty minutes. Twenty hours at room temperature. 4 = Complete agglutination. 0 = No. agglutination

PH VALUES	DILUTION OF ANTIGEN		
	1000 p. p. m.	500 p. p. m.	250 p. p. m.
2.2	3	3	2
2.4	4	4	4
2.6	4	4	4
2.8	4	4	4
3.0	4	4	4
3.2	4	4	4
3.4	4	4	4
3.6	3	4	3
3.8	3	2	2
4.0	2	2	0
4.2	1	0	0
4.4	0	0	0
4.6	0	0	0
4.8	2	1	0
5.0	3	2	1
5.2	1	1	0
5.4	0	0	0
5.6	0	0	0
5.8	0	0	0
6.0	0	0	0

at an hydrogen ion concentration of 3.6×10^{-5}N and another, apparently non-specific, at a concentration of 1.1×10^{-3}N.

It is probable that in spite of the complexity of the bacterial cell a comparatively small number of colloids are responsible for the reactions incident to agglutination.

Marked differences are to be observed in the agglutination of capsulated and non-capsulated bacteria due apparently to the

inhibiting influence of the capsular material itself. This is particularly well shown in the difficulty encountered in producing agglutination of Friedlander's bacillus. That this difficulty is due to the capsule was shown by Porges (1905), who succeeded in agglutinating the organism after hydrolysis of the capsule. Streit (1906) found that Friedlander's bacillus grown at 8°C. was drier, produced less capsular material and was more easily agglutinable than when grown at blood heat. Beham (1912) showed that agglutinin formation takes place in animals injected either with capsulated or non-capsulated strains, but can best be demonstrated with the occasional capsuleless cultures developed.

Inagglutinable strains of non-capsulated bacteria which are usually readily agglutinable have been frequently described. Weeney (1899) reported decided differences in agglutinability among typhoid races, particularly those from bile. Rodet (1899) isolated from the spleens of three typhoid cadavers bacteria which were not agglutinable when first cultivated, but finally became so. This experience was duplicated by Sacquépee (1901). Bail (1902) claimed that typhoid cells from the peritoneal exudate of a guinea pig showed decreased agglutinability. Cole (1904) found that inagglutinable bacteria bind less agglutinin than agglutinable bacteria. Kirstein (1904) cites many instances of inagglutinable bacteria. Ficai (1912) emphasized that freshly isolated vibrios do not agglutinate as well as the older laboratory strains. Reimer (1913) demonstrated a definnite diminution of agglutinability in typhoid grown on alkaline agar. McIntosh and McQueen (1914) found that an inagglutinable strain of typhoid had normal antigenic powers in production of anti-typhoid serum, and that it absorbed agglutinins though it did not agglutinate.

On the other hand bacterial cultures are frequently noted that are hyper-agglutinable. Kirstein (1904) found that typhoid bacilli when grown on asparagin agar became spontaneously agglutinable in salt solution. Neisser and Friedmann (1904) and Bechhold (1904) concluded that spontaneously agglutinating bacteria lack some substance, perhaps a protein, that is responsible for the inhibition of flocculation in normal cultures.

Friedberger and Lürrssen (1905) found spontaneous agglutination to be identical in appearance with serum agglutination. They also noted the important fact that it takes place only in the presence of salts, not in distilled water. Porges and Prantschaff (1906) showed that bacteria that were spontaneously agglutinable still combined with agglutinin. Porges (1906) also showed that an excess of agglutinating serum inhibits the agglutination of spontaneously agglutinable strains. McGregor (1910) found that meningococci grown for more than forty-eight hours became easily agglutinable, flocculation occurring in physiological salt solution or in normal serum. Kabeshima (1913) noted that old cholera bacilli frequently showed spontaneous agglutination. The agglutinins were adsorbed by these cells as well as by normal ones. Dilution of the salt prevented nonspecific or spontaneous agglutination. Verzai (1917) found a typhoid organism in urine which agglutinated spontaneously in physiological salt solution. Even after six weeks cultivation it still showed this peculiarity. He showed, however, that specific serum agglutination could be secured with a salt solution one-fourth the strength of physiological salt solution.

The antigen will contain varying quantities of products of growth and metabolism, and frequently also the products of more or less complete cell autolysis. These are the substances of import in the specific precipitation test as differentiated from agglutination. It seems probable that in some cases at least they are identical with the agglutinable constituents of the cells themselves. This is borne out by the experiments of Arkwright (1914). It is possible to wash bacteria until they lose a large part of their specific agglutinability.

The conditions under which bacteria are grown, the exact method of preparing the antigen, together with unexplained variations in the bacteria themselves, all may have their effect upon agglutinability. In a few cases this may even influence the development of agglutinins in the immunized animal. The experiments by Bordet and Sleeswijk (1910) on pertussis may be cited. Gay and Claypole (1913) found that socalled blood and bile strains of typhoid did not agglutinate with anti-typhoid

serum, although they absorbed it. They agglutinated however with the homologous antiserum.

Concentration of the antigen is of marked importance in the agglutination phenomenon. As will be noted later, this effect is manifested either when the precipitating agency is an electrolyte or a colloid, though in different ways. Porges (1906) with a suspension of typhoid secured partial agglutination in undiluted antigen by 30 per cent saturation with $(NH_4)_2SO_4$, but not in higher dilution. Examples of this effect with a meningococcus antigen are illustrated in table 1 with varying hydrogen ion concentration and in table 2 with $CaCl_2$. With cerous nitrate, in dilution of 1000 parts per million complete agglutination occurred in two ranges, from M/20 to M/40 and from M/640 to M/2560 and in 250 parts per million complete agglutination occurred at M/20 and in the range M/2560 to M/5120 (see table 3). These facts were well illustrated by Buxton and Rahe (1909). These investigators differentiated clearly between two distinct types of flocculation of colloids; the electrolyte type in which dilution of the colloid has no effect upon the flocculation

TABLE 2

Agglutination of meningococcus by calcium chlorid. Effect of dilution of antigen. Suspension of washed meningococci in distilled water. Incubated at 56°C. for twenty hours

DILUTION OF $CaCl_2$	STRAIN OF ANTIGEN					
	55		59		60	
	1000 p. p. m.	500 p. p. m.	1000 p. p. m.	500 p. p. m.	1000 p. p. m.	500 p. p. m.
M/2	0	0	0	0	0	0
M/4	0	0	0	0	0	0
M/8	0	0	0	0	0	0
M/16	0	0	0	0	1	0
M/32	2	0	0	0	3	3
M/64	3	2	0	0	4	4
M/128	3	2	0	0	4	4
M/256	1	0	0	0	4	2
M/512	0	0	0	0	1	0
M/1024	0	0	0	0	0	0
M/2028	0	0	0	0	0	0

limits, and there is no relation between the concentration of the electrolyte and the concentration of the colloid; and the colloidal type resulting from the flocculation of one colloid by another of opposite sign, in which the flocculation limits are dependent upon the relative concentration of the two colloids, i.e., if either colloid is much in excess there is no flocculation. Miller (1909) observed that the zone of inhibition of sera contianing so-called agglutinoid varies with density of the bacterial suspension, the more concentrated the antigen the lower the dilution of the

TABLE 3

Agglutination of meningococcus by cerous nitrate. Effect of dilution of antingen. Suspension of washed meningococci in distilled water. Incubated at 56°C. for twenty hours

DILUTION OF $Ce(NO_3)_3$	STRAIN OF ANTIGEN					
	56			60		
	1000 p. p. m.	500 p. p. m.	250 p. p. m.	1000 p. p. m.	500 p. p. m.	250 p. p. m.
M/20	4	4	4	4	4	4
M/40	3	3	1	4	4	3
M/80	2	2	1	3	2	1
M/160	0	0	0	0	0	0
M/320	0	0	0	0	0	0
M/640	4	0	0	0	0	0
M/1280	4	4	0	4	4	0
M/2560	4	4	4	4	4	4
M/5120	0	4	4	0	4	4
M/10240	0	0	0	0	0	3
M/20480	0	0	0	0	0	0

serum in which agglutination will occur. He concluded that this similarity to colloid flocculation rendered unnecessary the postulation of the existence of agglutinoids.

Beniasch (1911) calls attention to the fact that Hofmeister (1888) found a definite relationship between the size of the precipitating doses of the light metals and the dilution threshold characteristic of the protein itself. The minimal precipitating doses of such salts decreased with diminution of the concentration of the protein, while the maximal dose remained the same for all concentrations. Porges has shown a similar relationship for bacterial suspensions.

2. Adsorption of other colloids

It may be emphasized at this point that there are two somewhat variant current conceptions of bacterial agglutination. According to one view, bacterial cells determine the agglutination, they themselves repel or attract. The other conception is that a precipitation of some extraneous substance occurs, and the bacteria are carried down mechanically. It is not improbable that both conceptions are necessary to a complete explanation of the phenomenon under differing conditions. From the foregoing discussion it is apparent that the phenomenon of adsorption is in either case quite fundamental in any consideration of agglutination.

a. Colloids which agglutinate per se. Attention has been called to the fact that antigen may adsorb either colloids or electrolytic ions or both. Let us first consider the adsorption of colloids.

The adsorption of colloids by antigen may influence the course of agglutination in any one of several ways. It may favor the agglutination directly or indirectly. It may cause agglutination *per se*, it may increase the adsorption of some other colloid, or it may increase flocculability by salts and other electrolytes. In some cases it may have the opposite effect, decreasing the adsorption of other sensitizing colloids or flocculating electrolytes.

Numerous examples of colloids which agglutinate bacteria *per se* have been described in the literature. One well marked characteristic of the agglutination by the interadsorption of two colloids may be noted. An excess of either colloid beyond a definite proportion interferes with or prevents agglutination. If one colloid, as antigen, be diluted, the precipitating colloid must also be diluted. This gives rise to a so-called irregular series of Bechhold. Among the substances which have been found to flocculate bacteria in this manner are certain of the aniline dyes, hydroxids of some metals, and possibly certain sera. Aniline dyes in aqueous solution in general behave as colloids and have been used for bacterial agglutination by sev-

eral writers. Blachstein (1896) suggested the use of chrysoidin as a specific precipitant for the cholera vibrio, though Engel (1897) found its action not to be specific. Malvoz (1896) found that *B. typhosus* was not agglutinated by this dye, though he notes that safranin and vesuvin do produce agglutination in dilutions as high as those shown by a potent serum. McIntosh and McQueen (1914) found that serum-agglutinable and non-agglutinable strains of typhoid were agglutinated by saffranin in dilutions up to 1 in 3000.

The hydrosols of metallic hydroxids usually agglutinate bacteria, as well as other hydrosols bearing opposite charges. Biltz (1904) arranged a large number of hydrosols in two columns. When mixed in proper proportion those of one column would agglutinate those of the second. He also showed that the same type of irregular series occurs with bacteria and serum. It had previously been noted that dilute solutions of salts of iron, aluminium, cerium, etc., showed the characteristic colloidal irregular series as a result of the hydrolysis of the salt.

There are occasional records of other colloids showing an agglutinating action. For example, Trumpp (1898) succeeded in agglutinating cholera vibrios with various gum and starch solutions. It has already been noted that Bordet (1899) first showed the action of the agglutinin to be a sensitization of the cell to the action of salt. There is some evidence however, that the agglutinin may itself cause agglutination. For example, Porges (1906) dialyzed both typhoid bacteria and a potent antiserum until they were chlorin free and found agglutination would occur with a 1–10 dilution of serum but not in higher dilution unless salt was present. A similar fact is shown in chart I, though it is probable in this case that the undialyzed immune serum contained sufficient salt to cause agglutination of the sensitized cells.

Apparently the socalled specific agglutinins are colloids, which are taken up by bacteria in accordance with the laws of adsorption. Dreyer and Douglas (1910) studied the absorption of agglutinins by bacteria and the application of physico-chemical laws thereto. They determined that if an agglutinating

serum in different concentrations is treated with a constant number of bacteria, the amount of bound agglutinin from successive increments of serum progressively decreases and finally sinks to zero.

The amount of adsorption of one colloid by another may be markedly influenced by the presence of other substances in solution. In some cases it is possible to bring adsorbed substances back into solution by the addition of proper solutes, and thus to demonstrate the reversibility of the union. This reaction has been well worked out for hemolytic amboceptor by Kosakai (1918). He showed that this substance was removed from sensitized blood cells by an isotonic solution of sucrose, glucose, or lactose, Furthermore, it was found possible to sensitize the red cells with amboceptor, and by washing thoroughly, remove most of the amboceptor from the cells. In this way a highly purified amboceptor was secured. Apparently this has not been attempted with bacterial agglutinins, but it is entirely possible that they might be secured by a similar procedure. If so, it would constitute an excellent method for securing material for a more accurate study of their characteristics.

It has already been emphasized that the principal action of the adsorbed agglutinin is to render the sensitized cell flocculable by electrolytes. This was clearly shown first by Bordet (1899) and verified by Joos (1904) and many other investigators. This fact will be discussed more at length under the heading of electrolyte adsorption.

b Colloids which influence adsorption of other colloids. In some cases, the result of adsorption of specific agglutinin is decidedly more complex. It has been shown, for example, that adsorption of agglutinins may lead to greatly increased adsorption of certain non-specific serum proteins which in turn increase the adsorption of ions. The specific agglutinins sensitize to the serum protein, and this to the ions. Dean (1912) prepared socalled "complement fractions" from guinea pig serum by the method of Liefmann, and demonstrated that the action of anti-sheep (red blood corpuscles) sera or anti-typhoid sera were greatly increased in potency by the globulin (mid-piece) fraction.

Still more complex apparently is the phenomenon of conglutination. In this case the adsorption of the specific agglutinin increases the adsorption of a second colloid of ox serum, the conglutinin, which in turn sensitizes to a third colloid, complement, which is adsorbed and apparently sensitizes to the action of electrolytes. Muir and Browning (1906) found that the ability of anti-ox corpuscle serum from the rabbit to agglutinate ox red blood cells was greatly increased by fresh ox serum. Bordet and Gay (1906) also noted the marked agglutinative power of ox serum for sensitized red cells. This property was lost by heating the ox serum to 56°, but was restored by the addition of the complement. Bordet concluded that three distinct factors are involved; the action on the corpuscles of first, the specific serum, second the ox colloid, and third the fresh serum or alexin. Bordet and Streng (1909) named this ox serum constituent, conglutinin, and concluded that it did not need to be fixed on the cells that were conglutinated. Streng (1909) extended conglutination studies to bacteria. He found that by the use of ox serum and complement he could secure marked agglutination of bacteria in a dilution of the homologous antiserum by itself too weak to produce any trace of agglutination. When dialyzed, the agglutinin was precipitated with the globulin. Rankine (1910) also separated agglutinin and conglutinin. Hirvisalo (1913) noted that socalled exudate bacteria were vigorously clumped by normal cattle serum which had been deprived of normal agglutinins by previous treatment. This does not occur with the "native" typhoid; and evidently involves a conglutinin reaction.

The phenomenon of conglutination should not be confused with the co-agglutination of Bordet and Gengou (1911). These authors found that when a mixture of antigen and homologous antibody are added to a suspension of guinea-pig corpuscles, the latter are agglutinated. The sera used were heated to 56°C. to destroy the alexin. This reaction was obtained in all the antigen-antibody systems used, though it occurred only if a considerable excess of antigen was present and was best shown when the corpuscles were mixed with the antigen and the antibody then added.

These tests evidently show that corpuscles may adsorb specific substances from cultures which render them susceptible to agglutination.

c. Colloids which desensitize. The adsorption of a colloid may render the suspensoid less sensitive to various ions, that is, it may tend to desensitize. Bechhold (1904) for example showed that gelatin added to mastic suspension inhibited agglutination. From this he concluded that bacteria behave like suspensions possessing an albumenlike envelope, which protects the bacteria from flocculation with the salts of alkaline metals, for he found that the filtrate from twelve-day typhoid culture inhibited mastic flocculation. However, neither sensitized nor unsensitized cells changed their agglutinability in the presence of gelatin. Porges and Prantschaff (1906) concluded that spontaneously agglutinating bacteria lack the suspending action of proteins. Tulloch (1914) used 10 per cent alcohol and 1 per cent gelatin to determine the effect of differences in intrinsic pressure and internal friction on sensitized bacteria, but concluded that they were without influence.

3. Adsorption of non-electrolytes

The adsorption of non-electrolytes (not colloidal) may in some cases bring about agglutination. Malvoz (1897) found that suspensions of typhoid bacilli in distilled water were agglutinated by formalin, hydrogen peroxide and strong alcohol, but not with chloroform or phenol. He concluded that specific differences could be demonstrated between *B. typhosus* and *B. coli* by formaldehyde agglutination. His students, Lambert and Bossart (1898), could find no chemical substance that like cholera serum agglutinated specifically. Remy (1900) concluded the Malvoz formaldehyde agglutination was not strictly specific. A review of these articles seems to lead to the conclusion that in many of these instances the immediate action of the non-electrolyte is a denaturation. Gelatin, for example, is changed from a hydrophilic colloid to a suspensoid, easily flocculated, by the addition of formaldehyde. Apparently studies of bacteria

free from salts have not been made to determine these interrelationships. In this connection it is of interest to note that in certain types of chemical reaction Bayliss (1911) has found adsorption to be an essential preliminary.

4. Adsorption of electrolytes

a. Hydrogens ions. Most important as the apparent immediate cause of agglutination is the adsorption of cations by the bacteria. Studies thus far made show little effect of various anions, though that they are entirely without influence is scarcely probable. However, the influence may be disregarded for purposes of a general discussion.

We may, for convenience, discuss this effect under the heading of concentrations of hydrogen ions, and of ions of univalent, divalent, trivalent and polyvalent bases.

Bechhold (1904) noted that acids are very active in producing agglutination, the most powerful being those most strongly dissociated, such as HCl. The weak amidobenzoic acid flocculates poorly, while acetic acid is intermediate in its action. He found that M/1000 HCl or H_2SO_4 agglutinated unsensitized and M/2000 sensitized bacteria. With ortho-amido-benzoic acid 5M/1000 was required for both sensitized and unsensitized cells. Michaelis (1911) found that in a series of solutions with varying hydrogen ion concentration but constant salt content he secured flocculation with *B. typhosus*, at a hydrogen ion concentration of 4×10^{-5} N. Beniasch extended these observations to a large number of bacteria. For *B. typhosus* he found maximum flocculation at $P_H = 4.45$, though some variation with different strains was detected. In the *B. enteritidis* series two subgroups were described with optima at $P_H = 3.86$ and $P_H = 2.66$. With *B. coli* and *B. dysenteriae* he could detect no acid agglutination between $P_H = 7$ and $P_H = 2$. He concluded that the acid agglutinable substance of the bacterium is identical with the substance agglutinated by the specific serum. These findings of Michaelis and Beniasch have been verified by many subsequent studies. Michaelis and Davidsohn (1912) concluded that specific

serum agglutination could not be explained upon the basis of affinity due to electric charges. They found that the more concentrated the agglutinin, the less was the agglutination dependent upon the hydrogen ion concentration. Ostwald (1913) and Landsteiner (1913) urge that Michaelis and Davidsohn (1911) do not prove their point and have not invalidated the colloidal theory of immunity reactions. Krumwiede and Pratt (1912) also note that specific serum agglutination occurs over a wide range of P_H values. They found, however, that sensitized bacteria would agglutinate in tube 1 of a Michaelis series and to a much higher serum dilution than with salt solution. McIntosh and McQueen (1914) showed that typhoid bacilli agglutinable with serum as well as non-agglutinable strains were both clumped by a hydrogen ion concentration of 3.5×10^{-5} to 8×10^{-5} N. They contend that this does not support the view of Beniasch that agglutination by acid and serum depend upon the same factors.

Gillespie (1914) used one of the Michaelis series with pneumococci, and found decided salt interference with agglutination by acid in some cases. Young cultures of type I gave a narrow zone of agglutination with optimum hydrogen ion concentration of 5.5 to 11×10^{-4} N; ten strains of type II reacted to 22×10^{-4}. Ten strains of atypical pneumococci showed great variation. The atypical forms were uniformly lacking in salt susceptibility. Attention may be again called to table I, showing the range of acid agglutination with the meningococcus. In summary, it may be stated that although numerous attempts have been made to use acid agglutination as a specific test for the differentiation and recognition of bacteria, the method has apparently not found favor, and has not replaced the use of specific antisera.

b. Univalent cations. Bechhold (1904) regards univalent ions of the alkaline metals as of little significance in the agglutination of unsensitized bacteria. Mastic suspension, which is more sensitive, he found flocculated by high concentrations, as for example, M/1 NaCl. Typhoid cells, sensitized and washed, were flocculated by 0.025 N NaCl. Similar results were

secured by Neisser and Friedemann (1904). Crenderopoulo and Amos (1904) showed evidence of antagonistic action of sodium and potassium salts. Eisenberg and Volk (190–) studied the effect of various salts on agglutination of sensitized cells, and concluded that sodium chlorid favored, while potassium chlorid inhibited such agglutination. Porges and Prantschoff (1906) dialyzed spontaneously agglutinable typhoid organisms, and found that this treatment somewhat decreased their agglutinability with salts. A trace of agglutination was secured with N/8 NaCl and complete agglutination with N/2 NaCl. KCl, KNO_3 and $NaNO_3$ all required equivalent concentrations. Porges (1906) tested unsensitized cholera bacteria with $(NH_4)\ SO_4$ and secured partial agglutination with 20 and 30 per cent saturation and complete with 40 per cent. Typhoid bacilli showed partial agglutination at 40 per cent saturation and complete agglutination at 50 per cent; *B. coli* gave partial agglutination at 40 per cent and complete agglutination at 60 per cent. Friedlander's bacillus was agglutinated partially at 60 per cent and completely at 70 per cent. It may be noted that about the same series would be secured by rating these bacteria on the basis of ease of agglutination with specific sera.

Verzar (1917) studied the reaction of a typhoid strain showing socalled spontaneous agglutination in physiological salt solution. He found that by diluting the salt solution to one half or one quarter its strength, he no longer secured spontaneous agglutination but could get specific agglutination with antisera.

Porges (1906) mixed dialyzed bacteria and dialyzed antiserum 1–100 and determined the concentration threshold of numerous cations necessary for flocculation. With NaCl, KCl, KNO_3 NH_4NO_3 it was M/500, with Na_2SO_4 M/1000, K_2SO_4 M/1500, $(NH_4)_2SO_4$ M/1200. It will be noted that apparently the anion is without effect, and the cations Na, K and NH_4 are about equipotent.

Tulloch (1914) studied the effect of the electrolyte upon the titre of a serum. He tried NaCl, NaF, Na_2SO_4, Ka_2SO_4 Na_2HPO_4, $BaCl_2$ and Na_3Cit, equimolecular with 0.9 per cent NaCl; all caused agglutination with dilution of 1–8000 of serum, and

not 1–16,000. Salts which hydrolize to produce OH ions do not agglutinate. Chart I gives the results secured with various concentrations of antimeningococcic serum, sodium chlorid and

TABLE 4

Agglutination of meningococcus cells sensitized with immune serum and washed. Antigen no. 60. Turbidity 500 parts per million. Incubated at 56°C. twenty hours

NaCl	AGGLUTINATION	$CaCl_2$	AGGLUTINATION	$Ce_2(NO_3)_3$	AGGLUTINATION	SERUM	AGGLUTINATION
M/2	3	M/2	4	M/20	4	1/4	4
M/4	3	M/4	4	M/40	?	1/8	3
M/8	3	M/8	4	M/80	0	1/16	1
M/16	4	M/16	4	M/160	0	1/32	0
M/32	4	M/32	4	M/320	0	1/64	0
M/64	3	M/64	4	M/640	0	1/128	0
M/128	3	M/128	4	M/1280	0	1/256	0
M/256	0	M/256	4	M/2560	0	1/512	0
M/512	0	M/512	3	M/5120	3	1/1024	0
M/1024	0	M/1024	2	M/10240	4	1/2048	0
M/2048	0	M/2048	0	M/20480	1	1/4096	0
M/4096	0	M/4096	0	M/40960	0	1/8192	0

TABLE 5

Agglutination of meningococci with ammonium sulfate. Comparison of sensitized and nonsensitized cells. Strain no 55.

CONCENTRATION $(NH_4)_2SO_4$	SENSITIZED CELLS	UNSENSITIZED CELLS
2M	4	4
M/1	4	4
M/2	3	2
M/4	3	1
M/8	3	1
M/16	4	0
M/32	4	0
M/64	4	0
M/128	4	0
M/256	2	0
M/512	0	0
M/1024	0	0

meningococcus antigen. It is evident that an excess of sodium chlorid inhibits the specific agglutination. The explanation of the form assumed by the area showing complete agglutination

is apparently not a simple one. Strangely enough in certain concentrations of NaCl a decrease in the concentration of the serum increases the agglutination.

Table 4 shows the behavior of sensitized washed meningococci in the presence of different dilutions of various salts.

Table 5 shows the agglutination of both sensitized and unsensitized washed meningococci with ammonium sulphate.

3. Divalent cations

Bechhold (1904) found divalent bases stronger as agglutinating agencies than univalent ones. The salts of the alkaline earths such as barium chloride acted on mastic but not on normal bacteria, sensitized bacteria were flocculated in a dilution of 5 M/1000. Zinc sulfate and zinc nitrate agglutinated sensitized bacteria to a dilution of M/1000 and unsensitized bacteria to M/100. Other divalent metals showed high agglutinating power, varying from M/10,000 with sensitized to M/400 with unsensitized cells. Malvoz (1897) secured agglutination of typhoid bacilli in distilled water with 0.7 parts per thousand of mercuric chlorid. Crenderopoulo and Amos (1904) found that sensitized cholera vibrios agglutinated well with $CaCl_2$ 1 part to 10,000, this being five times as dilute as the effective solution of NaCl. Porges (1906) calculated that magnesium ions were about ten times as potent as univalent ions in producing flocculation of sensitized typhoid cells, the threshhold values being for $MgCl_2$ M/7500, for $Mg(NO_3)_2$ M/5000 and for $MgSO_4$ M/10,000. He argues that inasmuch as magnesium salts will not salt out this organism from normal suspensions, the mechanism must be different with sensitized bacteria.

Liefmann (1913) attempted to find specific differences among organisms by "salting out," particularly with $MgSO_4$. Paratyphoid bacilli of the Gärtner type he found to be flocculated by 80 per cent saturation, but neither *B. typhosus* nor *B. coli;* the latter form was usually not salted out even with 90 per cent concentrations. I have found no record of agglutination of unsensitized bacteria by salts of calcium. I was therefore sur-

prised to secure with meningococcic antigen the results given in table 2. It will be noted that with some strains a zone of complete agglutination at about M/64 is manifest. Either excess or deficiency of calcium ions may apparently inhibit flocculation. It is possible that other bacteria will show similar results upon further study.

4. Trivalent bases

Bechhold (1904) found the trivalent bases to be particularly powerful agglutinants. Iron and aluminium sulfate were found to flocculate in dilutions of M/10,000, fives time as dilute as a solution of HCl giving similar agglutination. Bechhold secured a very good example of an irregular series with both sensitized and unsensitized bacteria and $Fe_2(SO_4)_3$, and also with sensitized bacteria and $PtCl_4$. These irregular series were observed only with trivalent salts that showed strong hydrolysis, the colloidal iron oxid in the inhibition zone apparently preventing the flocculation. Neisser and Friedemann (1904) showed that when a cation shows a variable valence in its salts, its agglutinative power is in accorddance with the higher valence. Burton and Rahe (1909) also emphasize that with salts of the trivalent metals, on account of hydrolytic dissociation there is a combination of the electrolyte type and colloidal type of flocculation giving rise to irregular series.

Table 3 illustrates the mixed agglutination series secured with meningococci and cerous nitrate.

5. Polyvalent bases

Bechhold (1904) used $PtCl_4$ finding its threshhold at about five times the concentration of iron. Tulloch (1914) found that $TaCl_5$ gave a zonal agglutination. In some of my own work I have found that iridium chloride, $IrCl_4$, gives a regular agglutination series, and in relatively high dilutions.

Perhaps in a distinct category should be noted the precipitating power of certain organic salts particularly salts of the alkaloids. Sabrazes and Brenques (1899) noted that salts of quinine and atropine cause agglutination.

6. Relation of valence to power of agglutination

Bechhold (1904) first emphasized that with bacteria as with proteins, the power of a cation to cause flocculation increases with the valence. The same observation has been made by many subsequent writers. One of the best demonstrations of this fact is that of Tulloch (1904) who found that with sensitized cells NaCl and KCl gave agglutination to 0.002 N, while $CaCl_2$, $StCl_2$, $BaCl_2$ and $MgCl_2$ were all active up to 0.0004 N. He agrees with previous writers that the agglutinating activity of an electrolyte depends on the valency of the cation. He compared the effect of NaCl, $BaCl_2$ and $LaCl_3$ on sensitized cells with the following results. The highest dilution of the salt showing agglutination is given.

Sensitized.....	NaCl 0.004 N	$BaCl_2$ 0.0004	$LaCl_3$ 0.00003
Unsensitized.....	NaCl 0.004 N	$BaCl_2$ 0.0004	$LaCl_3$ 0.00003

Tulloch concluded that the law of increased activity of cations with increase in valence, $1 : x : x^2$ as suggested by Linder and Picton (1895) held at least, approximately. Salts with polyvalent cations behave differently from those of mono and divalent cations. Apparently they are as active in the agglutination of unsensitized as sensitized bacteria.

7. Denaturation or sensitization by non-colloids

Several instances are on record of the sensitization of bacteria to the action of an electrolyte by the action of a non-electrolyte. For example, Bechhold (1904) found that bacteria treated with alcohol and washed were agglutinated by strong NaCl, and by even relatively weak dilutions of divalent cations. The same author also showed that bacteria could be sensitized by contact with lead acetate or uranyl acetate followed by washing. Imai (1912) studied the effect of osmium on bacterial agglutination and found the osmated bacteria more strongly agglutinated than normal.

Tulloch (1914) sensitized bacteria with aluminium and studied the flocculation with various salts. The agglutination thresh-

hold of the sensitized bacteria for the various salts were as follows: NaCl N/200; $BaCl_2$ N/2000; $LaCl_3$ N/5000; Ce_2Cl_6 N/10,000; $AlCl_3$ N/500 negative zone and N/1000 to N/10,000 positive. With unsensitized cells the results were as follows: NaCl, all negative; $BaCl_2$, all negative; $LaCl_3$ N/5000; Ce_2Cl_6 N/20,000; $AlCl_3$ N/1000 negative and N/2000 positive; N/20,000 positive and N/40,000 negative.

8. Graphic representation of action of mixtures

Probably one of the fertile fields for investigation will prove to be a study of the effect of varying two or more of the constituents of the mixture which under certain conditions may cause

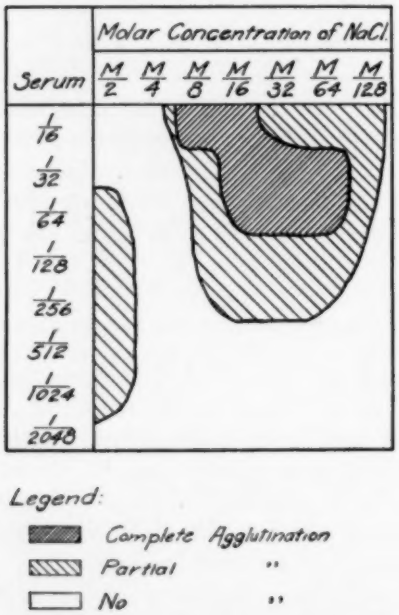

CHART I. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF IMMUNE SERUM AND SODIUM CHLORID

Antigen no. 60. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

agglutination. This has been done for typhoid bacteria, anti-typhoid sera and various electrolytes by several writers.

Porges (1906) dialyzed typhoid bacteria and the homologous antiserum until chlorin free. With serum in dilution of 1–10

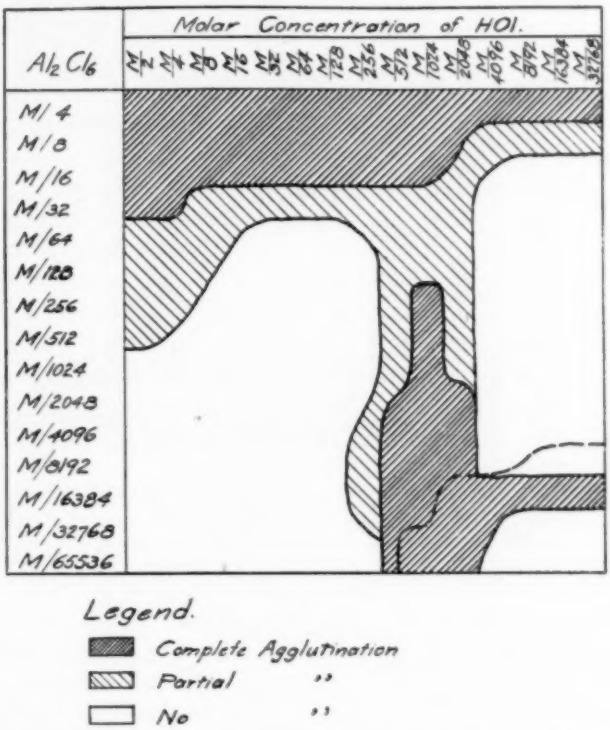

CHART II. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF ALUMINIUM CHLORID AND HYDROCHLORIC ACID

Antigen no. 55. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

he secured agglutination without NaCl, but not in any higher dilution. N/500 NaCl gave partial agglutination with a dilution of 1–500 of serum and N/50 NaCl, partial agglutination with 1/5000 dilution of serum. The general rule deduced was

that with increasing amounts of agglutinin the amount of salt necessary to agglutination decreases. Tulloch (1914) studied the relationships between concentration of serum and of elec-

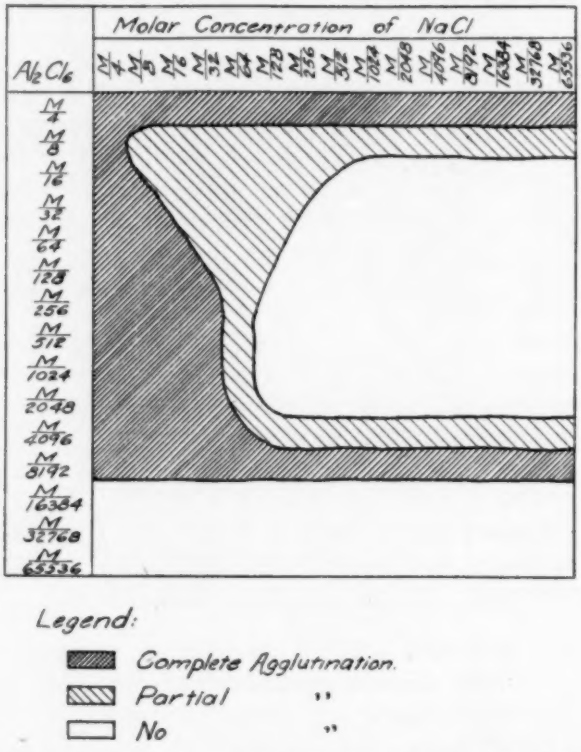

CHART III. AGGLUTINATION OF MENINGOCOCCUS BY VARIOUS MIXTURES OF ALUMINIUM CHLORID AND SODIUM CHLORID

Antigen no. 59. Turbidity 500 parts per million. Incubated at 56°C. for twenty hours.

trolytes and came to similar conclusions. He used both NaCl and $BaCl_2$.

Walpole (1914) has introduced a valuable diagrammatic method of studying the effect of varying the concentration of two different substances on the flocculation of a sol, which he applied to

the effect of varying gelatin and acid concentration upon the flocculation of an oil emulsion. An understanding of the effects of various combinations upon bacteria will be facilitated by pursuit of a similar method.

Chart I illustrates this method as applied to variable antiserum and variable sodium chlorid concentrations on meningococcic antigen.

Chart II plots the agglutination of a meningococcus antigen by varying proportions of aluminium chlorid and hydrochloric acid. This chart shows zones of agglutination by hydrogen ions, by aluminium ions, and by the aluminium hydrate, and zones of increased agglutination due to the combined action of hydrogen and aluminium ions. Areas are also shown in which there is inhibition by the aluminium of acid agglutination, and others in which the acid inhibits the action of the aluminium.

Chart III illustrates similar facts for various mixtures of aluminium chlorid and sodium chlorid.

CONCLUSIONS

There is ample justification for the conclusion that bacterial agglutination is a colloidal phenomenon that can best be studied in the light of the modern work on colloidal and physical chemistry. There is real need for intensive study of the complex inter-relationships of the factors which govern this phenomenon that they may be more clearly understood, and the diagnostic use of agglutination put upon a more scientific, rather than a purely empirical basis.

REFERENCES

ARKWRIGHT, J. A. 1914 On the presence in an emulsion of *Bacillus typhosus* of two different substances which are agglutinable by acids, and their relation to serum agglutination. Zeitschr. f. Immun. Orig., **22**, 396.

BECHHOLD, H. 1904 Die Ausflockung von Suspensionen bezw. Kolloiden und die Bakterienagglutination. Zeitschr. f. physik. Chemie, **48**, 385.

BEHAM, L. M. 1912 Die agglutinatorischen Eigenschaften der Kapselbacillen und die Anwendung der Serumagglutination bei den Trägern von Kapselbacillen. Cent. f. Bakt. Abt. I, Orig., **66**, 110.

BENIASCH, M. 1911 Die Säureagglutination der Bakterien. Zeitschr für Immunitätsforschung Orig., **12**, 268.

BILTZ, WILHELM 1904 Ein Versuch zur Deutung der Agglutinierungsvorgänge. Zeitschr. f. physik. Chemie, **48**, 615–623.

BLACHSTEIN 1896 Ueber das Verhalten des Chrysoidins gegen Choleravibrionen. München. med. Wochnschr. **43**, 1067.

BORDET, L., AND STRENG, O. 1909 Les Phénomenes d'Adsorption et la Conglutinine du Serum de Boeuf. Cent. f. Bakt. Abt. I, Orig., **49**, 260.

BORDET, J., AND SLEESWIJK 1910 Sérodiagnostic et variabilité des microbes suivant le milieu de culture. Annal. de l' Inst. Past., **24**, 476.

BOSSART 1898 Etude sur l'agglutination comparée du vibrion cholérigue et des microbes voisins. Am. de l'Inst. Past., **12**, 857.

BUXTON, B. H., AND RAHE, A. H. 1909 Effect of dilution upon the flocculation of colloids. Journ. of Medical Research **20**, 113.

BUXTON, B. H., AND RAHE, A. H. 1909 Effect of dilution upon the flocculation of colloids. II. Journ. of Med. Research, **20**, 311–325.

CHICK, H., AND MARTIN, C. J. 1912 On the heat coagulation of proteins. Part IV. The conditions controlling the agglutination of proteins already acted upon by hot water. Journ. Physiol., **45**, 261.

CRENDIROPOULO, MILTON, AND AMOS, MISS B. SHELDON 1904 On agglutination of vibrios. Journ. of Path. and Bact., **9**, 260.

DEAN, H. R. 1912 On the mechanism of complement fixation. Journ. of Hygiene, **12**, 259.

DEAN, H. R. 1912 On the factors concerned in agglutination. Proc. Royal Soc. of London Ser. B. Biol. Sci., **84**, 416.

DREYER, G., AND DOUGLAS, S. C. 1910 On the absorption of agglutinin by bacteria and the application of physico-chemical laws thereto. Proc. of the Royal Soc. B., **82**, 185.

DREYER, G., AND DOUGLAS, C. 1910 The velocity of reaction in the adsorption of agglutinins, trypsin and sulfuric acid by animal charcoal. Proc. Roy. Soc. B., **82**, 168.

EISENBERG, PHILIPP 1906 Weitere Untersuchungen über den Mechanismus der Agglutination und Präzipitation. Cent. f. Bakt. Abt. I. Orig., **41**, 96, 240, 358, 459, 539, 651, 752, 823, 864.

ENGELS 1897 Die Diagnose der Vibrionen mit Hilfe des Chrysoidins Cent. f. Bakt. 21, 81.

FICAI, GIUSEPPE 1912 Fenomeno paradosso del l'agglutinazione del vibrione colerigeno. Pathologica, **4**, 69.

GAY, F. P., AND CLAYPOLE, E. F. 1913 Induced variations in the agglutinability of *Bacillus typhosus* J. A. M. A., **60**, 1141.

GILLESPIE, L. J. 1914 The acid agglutination of Pneumococci. Journ. of Exp. Medicine, **19**, 28–37.

GROTE, L. R. 1913 Ueber die praktische Verwertbarkeit der Säureagglutination nach Michaelis. Centr. f. Bakt. Abt. I. Orig., **69**, 98.

GROTE, L. R. 1914 Zurkritik der Säureausflockung nach Michaelis, Cent. f. Bakt. Abt. I, Orig., **73**, 202.

HARDY, W. B. 1899 On the coagulation of proteid by electricity, Journ. of Physiol., **24**, 288.

HARDY, W. B. 1900 A preliminary investigation of the conditions which determine the stability of irreversible hydrosols. Proc. of Royal Soc. of London, **66,** 110.

HIRVISALO, K. F. 1913 Zur Agglutinationsresistanz der sogenannten Exsudatbakterien. Zeitschr. f. Imm., **17,** 449.

HOFMANN, F. B. 1914 Versuche über Benetzung, Emulsion, Agglutination und verwandte Erscheinungen. Zeitschr. f. Biol., **63,** 486.

JAFFÉ, RUDOLF 1912 Säure- und Normal agglutination der Typhus-Coligruppe, Arch. f. Hyg., **76,** 1.

JOOS, A. 1902 Untersuchungen über die Bedeutung der Salze bei der Agglutination, Zeitschr. f. Hygiene, **36,** 422.

KABESHIMA, T. 1913 Ueber den Spontanagglutination der Choleravibrionen. Saikin Gakuzassi, No. 216; Abs. Zeit. f. Immunitätsforschung, Ref., **8,** 792.

KIRSTEIN, F. 1904 Ueber Beeinflussung der Agglutinirbarkeit von Bakterien, insbesandere von typhusbacillen, Zeitschr. f. Hyg., **46,** 229.

KOSAKAI, M. 1918 The isolation, purification and concentration of immune bodies: A study of immune hemolysin. Journ. Immunol., **3,** 109.

KRUMWIEDE, CHAS., AND PRATT, JOSEPHINE 1912 Die Säureagglutination sensibilisierter Bakterien. Zeitschr. f. Immunitätsforschung, Orig., **16,** 517.

LANDSTEINER, KARL AND STANKOVIČ, RADENKO 1906 Ueber die Adsorption von Eiweiss körpern und über Agglutininverbindungen, Cent. f. Bakt. Abt., I, Orig., **41,** 108.

LANDSTEINER, K., AND V. JAGIČ, N. 1904 Ueber Analogien der Wirkungen kolloidaler Kieselsaure mit den Reaktion der Immunkörper und verwandter Stoffe. Wien. klin. Wchnschr., **17,** 63.

LANDSTEINER, K. 1913 Zur Frage der Spezifität der Immunreaktionen und ihrer kolloidchemischen Erklärbarkeit. Bioch. Zeitschr., **50,** 176.

LIEFMANN, H. 1913 Die Aussalzung des Bakterieneiweisses, ein Hilfsmittel der bakteriologischen Diagnostik. Zeitschr. f. Imm. Ref., **7,** 463.

LIEFMANN, H. 1913 Die Unterscheidung verwandter Bakt erienarten durch die Ausfällung ihres Eiweisses mittels konzentrierter Salzösungen. München. Med. Wochenschr., **60,** 1417.

MCINTOSH, J., AND MCQUEEN, J. M. 1914 The immunity reactions of an inagglutinable strain of *B. typhosus*. Journ. Hygiene, **13,** 409.

MACGREGOR, A. S. M. 1910 Immunity-phenomena in cerebro-spinal meningitis: Opsonins and agglutinins in their relation to clinical features, prognosis and therapy. Journ. Path. and Bact., **14,** 503.

MALVOZ, E. 1897 Recherches sur l'agglutination du *Bacillus typhosus*. Ann. de l'Inst. Pasteur, **11,** 582.

MALVOZ, E. 1899 Sur la presence d'agglutinines specifiques dans les cultures microbiennes. Ann. de l'Inst. Pasteur, **13,** 630.

MICHAELIS, LEONOR 1911 Die Säureagglutination der Bakterien, insbesondere der Typhusbazillen. Deutsche med. Wochenschr., **37,** 969.

MICHAELIS, L. Physik. chem. d. kolloide. Richter-Kornayis Hdb., **2,** 391.

MICHAELIS, L., AND DAVIDSOHN, H. 1912 Die Abhängigkeit spezifischer Fällungsreaktionen von der Wasserstoffionenkonzentrationen, Biochem. Zeitschr., **47,** 59.

MILLER, F. C. L. 1909 Concerning so-called agglutinoids. Journ. Inf. Dis., **6**, 361.

MOORE, BENJAMIN 1917 The theory of immunity. Brit. Med. Journ., **2**, 842.

MUIR AND BROWNING 1906 On the action of complement as agglutinin. Journ. Hygiene, **6**, 20.

NEISSER, M., AND FRIEDEMANN, U. 1904 Studien über Ausflockungserscheinungen, Münchener Medizin. Woch., **51**, 465.

NEISSER, M., AND FRIEDEMANN, U. 1904 Studien über Ausflockungserscheinungen, II. Beziehungen zur Bakterienagglutination. München. Medizinische Wochenschr., **51**, 827.

OSTWALD, WO. 1913 Zur Frage nach der kolloidchem Analyse des Spezifitätsproblems, Biochem. Zeitschr., **48**, 225.

OSTWALD, WO. 1913 Die neuere Entwicklung der Kolloidchemie, Kolloidchem. Beihefte zur kolloidchem. Zeitschr., **4**, 1.

POPPE, DR. 1912 Die Saureagglutination der Bakterien der Paratyphus gruppe. Zeitschr. f. Immunitätsforschung, Orig., **13**, 85.

PORGES, O. 1905 Agglutinabilität der Kapselbakterien. Wiener, klin. Wochenschr., **28**, 691.

PORGES, O. 1905 Ueber die Beziehungen zwischen Bakterienagglutination und Ausflockungserscheinungen der Kolloide, Cent. f. Bakt. Abt. I. Orig. **40**, 133.

PORGES, O. 1909 Ueber Kolloide und Lipoide in ihren Beziehungen zur Immunitätslehre. Kraus and Levaditi, Handbuch der Technik und Methodik der Immunitätsforschung, **2**, 1136.

PORGES, O., AND PRANTSCHOFF, A. 1906 Ueber die Agglutinabilität von Bakterien, besonders des *B. typhi*, Centr. f. Bakt. Abt. 1, Orig., **41**, 466, 546, 658.

PRIESTLEY, HENRY 1917 The mechanism of the agglutination reaction. Journ. Hygiene, **15**, 485.

REMY, L. 1900 Contribution à l'étude de la fièvre typhoide et de son bacille, Ann. de l'Inst. Past., **14**, 555.

RIEMER 1913 Ueber Beeinflussung der Agglutinierbarkeit von Typhus bacillen durch den Alkaligehalt des Nährbodens. Münch. Med. Wochenschr., **60**, 908.

ROBERTS, MORLEY 1917 The theory of immunity. Brit. Med. Journ. 777.

ROST, FRANZ 1911 Die Verwertung der Säureagglutination zur Diagnose des Typhus. Cent. f. Bakt. Abt. 1, Orig., **60**, 324.

SABRAZES AND BRENGUES 1899 Agglutinines chimiques. Comp. rend. soc. Biol.

SACQUEPEE 1901 Annales de l'Inst. Past., **15**, 249.

SCHIDORSKY, H AND REIM, W. 1912 Die praktische Verwertung der Säureagglutination der Bakterien, Deutsch. med. Wochenschr., **38**, 1125.

SCHMIDT, P. 1913 Physikalisch-chemische Untersuchungen über die Serumagglutination, Arch. f. Hyg., **80**, 62.

SGALITZER, MAX 1913 Ueber Säureagglutination, Zeitschr. f. Hygiene u. Infektionskrak., **76**, 209.

STEPANOFF-GRIGORJEFF, J. J., 1912 Zur Frage der Säureagglutination nach L. Michaelis beim Abdominaltyphus und bei der Pestis humana. Russky Wratsch., **11**, 81; Abs. Zeitschr. f. Imm. Ref. **5**, 207.

STREIT 1906 Zur Frage der Agglutinierbarkeit von Kapselbacillen. Cent. f. Bakt., Abt. I., Orig., **40,** 709.

STRENG, O. 1909 Studien über das Verhalten des Rinderserums gegenüber den Mikroben. Cent. f. Bakt. Abt. I, **50,** 47.

TRUMPP 1898 Das Phänomenon der Agglutination und seine Beziehungen zur Immunität, Arch. f. Hyg. **33,** 70–144.

TULLOCH, Wm. J. 1914 The mechanism of agglutination of bacteria by specific sera. Biochemical Journ., **8,** 293.

VERZAR, FRITZ 1917 Ueber spontanagglutinierende Typhusbazillen, Cent. f. Bact., Abt. I, Orig., **70,** 161.

VOLK, RICHARD 1909 Über Agglutination. Kraus and Levaditi's Handbuch, **2,** 623–689.

WALPOLE, G. S., 1914 Diagrammatic co-ordination of phenomena relating to aggregation of sols, Biochemical Journal **8,** 170–192.

METHODS OF PURE CULTURE STUDY

PROGRESS REPORT FOR 1918 OF THE COMMITTEE ON THE DESCRIPTIVE CHART OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST, M. J. PRUCHA, AND K. N. ATKINS

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I. THE DESCRIPTIVE CHART AND ITS PURPOSE

Since 1905 there has been in existence, with occasional modification, a chart endorsed by the Society of American Bacteriologists for recording the characteristics of bacterial cultures. Recent literature, however, indicates that some confusion exists as to the purpose of this chart. This confusion was well illustrated at the 1917 meeting of the Society, the discussion called forth by the report of this committee showing that a number of bacteriologists have ideas as to the object of the chart different from those held by the present members of the committee. The confusion is natural, for each change in the membership of the committee has caused the chart to develop along slightly new lines; and even the early members of the committee have gradually changed their ideas as the result of later experience. It seems desirable, therefore, to restate the purpose of the descriptive chart.

The first bacteriologists to devise descriptive charts did so on individual initiative and apparently had in mind merely the concise description of their cultures preliminary to publication. An examination of the cards of Gage and of H. W. Conn[1] suggests that nothing further than this was expected of these two charts. Later, however, it was felt that uniformity in the cards used by

[1] For a description of these cards the reader is referred to a paper by H. A. Harding (1910).

different workers would make it possible to compare the results obtained in different laboratories. The Society, therefore, appointed a committee to prepare an official chart, and in its report this committee expressed itself as follows:

> The past literature of bacteriology abounds in such imperfect descriptions of organisms as to make their grouping, according to any system, impossible. This fact calls for the adoption of some scheme to which all descriptions shall conform, in order that no essential character shall be overlooked. The accompanying card is proposed for the recording of the characters of an organism. . . . . The cards can be filed like catalog cards, and arranged in accordance with the group number, thus bringing similar organisms together and rendering comparison easy.

This seems to imply that the chief objects intended to be served by the chart are first to secure uniformity in published descriptions and second to facilitate the grouping of bacteria according to their characteristics. Recent literature, however, reveals the existence of an idea not expressed in this report: namely, that the group number on this official card furnishes a more satisfactory method of characterizing an organism than its identification with a specific name occurring in the literature. The idea probably had its origin in the form of group number introduced with the Society card. Gage and Conn had both used a group number, but their group number consisted merely of four digits which could have suggested to no one that it constituted a complete description of a species. The Society group number, however, was simplified by leaving off those digits that indicated form and motility, replacing them with the genus symbol (according to Migula), which was placed before the series of digits. That this use of the genus symbol was not considered by the first committee to convert the group number into a method of characterising species is shown by the following statement which they made in regard to the group number:

> This system readily enables organisms having similar characters to be brought together and grouped about some central organism or type.

The only hint in this report that the group number might replace a specific name occurs a little later when the statement is made that according to this method of expression "Bacillus coli (Escherich) Migula becomes B.212.33310." This statement suggests to the reader, especially if that reader be a beginning student in bacteriology, that the string of digits 212.33310 is equivalent to the specific term *coli*. It naturally led to the impression on the part of the part of certain younger bacteriologists that the group number is nearly all-sufficient in describing a new species.

This point of view was strengthened by a paper from a member of this committee (Harding, 1910) on *Ps. campestris*, showing this organism apparently to have a constant group number. This work did not show that no other organism has the same group number, nor that all other organisms are so constant in physiological characteristics as is this particular species, nor indeed that this organism would have given such constant results if the technic had been varied; but there has been some tendency on the part of other bacteriologists to read into the paper a conclusion of this nature. The statement has even been made in one recent paper that the group number obviates the necessity of bacterial names.

On the other hand, various investigators, using other species of bacteria, have one by one criticised every point in the group number except that which refers to spore-production, showing that the same culture may give different results each time the tests are repeated. Futher criticisms have arisen from the biometric studies of bacteria, such variations having been observed among the different strains of a single species that no characterization of a species can be considered valid until the mean is determined around which the different strains vary.

These criticisms have sometimes been met by the statement that if the technic were sufficiently perfected and standardized, each strain would always prove to have the same group number, even though that group number might not express the characteristics of the species under all other possible conditions. An objection to this argument has been pointed out by Breed (1914)

in his work on the nitrate reduction of *B. coli*. He shows that the use of a standard medium, instead of distinguishing sharply between organisms with positive reactions and those with negative reactions, is likely to bring about variable results for those which grow poorly in the medium adopted. Recent work of the committee, moreover, shows very plainly that (in regard to nitrate reduction and acid production at least) no standard medium can be adopted which will give consistent results with all kinds of bacteria.

In brief, then, it may be said that the descriptive chart provides a uniform and concise method of recording nearly all of the ordinary and some of the unusual observations concerning the morphology and activities of an organism. Of the many characteristics listed on the chart, about a dozen were selected by former members of the committee as being the most important and were collected into a numerical expression called the group number. This group number is merely an abbreviated method of recording these characteristics, and must not be regarded as a sole means of characterizing species. Its value as a means of characterizing cultures, or strains, or species depends not only upon the wisdom with which these particular reactions have been selected but also upon the accuracy with which each individual characteristic can be determined. In both of these respects any group number proposed at present would necessarily be imperfect: first, because the diagnostic importance of the various characteristics differs among different groups of bacteria; and secondly because the methods for making the determinations have never been perfected and the results are correspondingly inaccurate. The problems of the committee, therefore, include the selection of the characteristics of greatest diagnostic value and the elaboration of methods which shall minimize mistakes. The committee is now studying the methods for working out the old group number; but, as a more permanent improvement, a new group number is being planned, less arbitrary and more logical than the one now on the card.

In addition to these weaknesses arising from our imperfect knowledge of bacterial activities and the methods of determin-

ing them, the card has not been revised frequently enough to contain the more recently devised tests for special groups of bacteria. Hence it is coming to be less and less used as a means of publishing descriptions of bacteria. In other words, it no longer helps to secure uniformity in bacterial characterization, the purpose for which it was originally intended.

Meanwhile instructors in bacteriology have realized the advantages of some such method of concise description in familiarizing students with the characteristics of bacteria. Some have used the official descriptive chart; but this chart was never intended for instruction purposes and is not designed to be put in the hands of beginning students. As a result other instructors have prepared descriptive charts of their own. Each of these charts has its own advantages and is undoubtedly better adapted to instruction than the old Society card; but for obvious reasons it would be better if a uniform style of chart were used in all institutions. The present committee, therefore, was instructed to prepare a chart especially adapted to use in bacteriological instruction: A new folder was prepared in response to these instructions and was presented at the 1917 meeting. It is now in print and is available to any teacher who wishes to try it out in his classes.

As the descriptive chart is now chiefly used for instructional purposes, it seems wisest to emphasize this use of it; but the committee does not wish to imply that an improved form of chart would be of no use in research work. The society card, indeed, has proved well adapted to certain types of research work in which a general survey is desired of the bacterial flora of some particular medium, preliminary to a later, more intensive, study of the individual species.[2] For some of this work the newer chart, although intended primarily for instruction, may prove more useful than the old form; but the old card is still available for use in this type of work whenever it is desired. Better still, for this purpose, would be a new card more nearly in accord with modern methods of characterizing bacteria, and

[2] Preliminary flora studies of this nature are well illustrated by the work of Conn, Esten and Stocking (1906) and by that of Harding and Prucha (1908).

specially adapted to research work. The committee hopes to prepare such a card in the future; but before that is done more attention must be given to the value of the various characteristics and to the methods of determining them.

The 1917 report of this committee (Conn. et al. 1918) contained a discussion of methods of pure culture study, designed primarily to accompany the chart for instruction. Further work has been done on some of these methods during the past year. A year ago it was expressed as the desire of the committee that these methods "be adopted as standard methods at the 1918 meeting, after such changes have been made in them as the year's use shows to be necessary." This is no longer urged. With the modifications given in the following pages, they are the best methods now known to the committee, and their use in instruction laboratories is strongly recommended; but as the results of criticisms of the 1917 report, together with the committee's further work during 1918, it seems wisest to regard them as provisional methods. No action is therefore asked on these methods and the committee desires to do further work on them.

The following pages contain a progress report on those methods that have been investigated during the past year, namely: Gram stain, acid production, and nitrate reduction. Free criticism of all matters discussed in this report, as well as the methods outlined in the preceding report, will always be welcome.

II. THE GRAM STAIN

At the last meeting of the Society one or two members criticised the classification of bacteria according to their ability to take the Gram stain, stating that many of the methods of making this test give irregular results and that the methods given in text books vary greatly from one another. The committee was asked to look into the matter. Gram originally gave no definite length of time for treatment in the different fluids; but as the results of the staining depend greatly upon the duration of each treatment, various investigators have felt it necessary to specify

definite periods of time. Unfortunately, however, scarcely any two investigators have agreed in the periods specified.

From two of the members who particularly objected to the lack of agreement between present methods a statement has been obtained as to the technic giving best results in their laboratories. The two methods thus obtained differ considerably: one method calls for a comparatively concentrated stain, a short period of staining and long decolorization, the other for a weaker stain, longer staining period and short decolorization. The former method is less time-consuming and the gentian violet solution it is claimed deteriorates more slowly; but upon comparison of the two methods no further differences have yet been observed. Both methods have been found to give clear-cut distinctions between the Gram-negative and Gram-positive organisms thus far studied. The committee realizes the advisability of adopting a standard technic, but feels that no method should be made standard without more investigation than has so far proved possible. Both methods are therefore given as capable of yielding good results; and the criticism of the Society upon them is invited.

Method 1

Gentian violet solution ..... 1 minute
(Stain prepared by grinding 5 grams in 10 cc. of 95 per cent alcohol in a mortar. Add 2 cc. anilin oil, distilled water 88 cc. Filter. Claimed to keep 3 to 4 months.)
Iodine solution (Lügol's) ..... 1 minute
(As usual: Iodine 1 gram, potassium iodide 2 grams, water 300 cc.)
Absolute alcohol ..... 2 minutes
Counter-stain ..... 30 seconds
(10 cc. of saturated alcoholic safranin in 90 cc. water.)

Method 2

Gentian violet solution ..... 3 minutes
(Anilin oil 3 cc., absolute alcohol 7 cc., water 90 cc. Shake and filter through moist filter. Add 2 grams of gentian violet and allow to stand 24 hours.)
Iodine solution (Lügol's, as above) ..... 2 minutes
Absolute alcohol ..... 30 seconds

Counter-stain:
Either Fuchsin (one part saturated alcoholic solution to nine parts of water) ..... 30 seconds
or Bismark brown (2 per cent, dissolved in hot water and filtered) ..... 2 minutes

Whichever method is used there are certain points in the technic that are important and must be insisted upon, especially if the test is being made by a beginner. The medium used must be recorded. Only young cultures should be used (eighteen to twenty-four hours old, except in case of unusually slowly growing organisms). Films should be on cover slips, made with distilled water, and in the portions examined organisms should be only one layer thick. Gentian violet and iodine solutions should each be removed by merely draining off the excess and blotting, before applying the next solution. Cover slips should be kept in constant agitation while in the alcohol and should then be transfered without washing to the counter-stain.

III. THE PRODUCTION OF ACID

Four places in the group number are devoted to the fermentation of sugars and glycerin. In the 1917 report the committee recommended studying this reaction in standard peptone broth. There are various possibilities of error inherent in this method, and it is not applicable to all bacteria. Accordingly a study has been made of the sources of error and the simplest methods of overcoming them.

Sources of error in determining acid production

One of the chief sources of error in determining acid-production comes from the weakness of the common methods of detecting and measuring acid. A change in the titre of any medium as the result of bacterial growth is not necessarily in the same direction as the actual change in H-ion concentration. This makes the use of the titration method unsatisfactory for detecting acid production. This source of error, however, may be overcome by the use of the proper indicators if the student has

a good comprehension of the principles of H-ion concentration. A simple but satisfactory method of using these indicators and interpreting the results was given in the 1917 report (pp. 122–124). The significance of these indicators and their use is discussed in more detail in the following pages.

Certain other sources of error are not so readily eliminated. Among them is the use of media in which the organisms in question make poor growth. If an organism does not grow well in a medium it is not correct to state that it is unable to produce acid from the sugar present in that medium. Certain organisms do not grow well in ordinary broth because they require more nitrogenous matter and a suitable medium for them should be found before adding the carbohydrate upon which their action is to be studied. Others require a different initial reaction from that of ordinary standard media. Others grow well only on solid media and give consistently negative results if tested in liquid media, merely because of their poor growth.

Another equally serious source of error is the fact that many bacteria may cause two simultaneous biochemical activities, one tending to raise the reaction, the other to lower it. The resultant change in reaction depends upon which of these two processes predominates, and the production of acid may often be entirely masked by the opposite tendency. This may often be the cause of variation in the results obtained upon repetition of the test with the same culture; and it may even cause an acid-producer to escape recognition entirely.

Error may also arise from impurities in the sugars used. Glucose is very often present as an impurity in other sugars, especially in those which are difficult to purify, but has also been observed in samples of sucrose and lactose. The presence of glucose can be detected by growing in it a known glucose fermenter that does not attack the sugar in question. This test should be made in all accurate work.

Media recommended

On account of these difficulties, it proves impossible to recommend any one standard medium. The formulae recommended

(J. Bact., **3,** 115, 116) are merely for routine use when the growth requirements of an organism are not known. If the organism grows poorly in these media, the results of the test are to be disregarded.

In connection with preliminary invigoration, the committee has already recommended (J. Bact. **3,** 118) dividing the organisms into four series according to their preference for 37° or 25°, and for liquid or for solid media. It is now further recommended that all organisms of series III and IV (i.e., growing well on the surface of agar but not in glucose broth) be tested for acid production by inoculating onto the surface of beef-extract agar (instead of broth) containing the carbonaceous substance to be tested. Bacteria not growing well in glucose broth or in beef extract agar must be tested in some medium in which they do make good growth. For them is recommended varying first the peptone content and then the acidity of the medium. The medium to which the sugar, glycerine or other substance is added should differ as little as possible from these standard media.

Methods of detecting acid production

For the reasons above discussed acid production should not be detected by means of titration. An indicator should be used whose range of color change covers the initial reaction of the medium used or else is slightly to the acid side of the initial reaction. (Thus brom cresol purple, with the range of $P_H = 5.2$ to $P_H = 6.8$ is the most satisfactory indicator to use with media of initial reaction of $P_H = 7.0$) Litmus can be used, but the results obtained with it are not sufficiently clear cut.

Indicator media are especially valuable now that we have indicators which are not attacked by bacteria, with turning points at the useful part of the P_H-scale. Litmus and methyl red are of but little use for this purpose because they are decolorized by bacteria; but the sulphone-phthalein indicators are not acted upon by bacteria and are just as satisfactory when mixed with the media before inoculation as afterward. The

addition of the minute quantities of these indicators necessary does not ordinarily influence the growth of the bacteria, and in most cases its influence can be disregarded.

The use of saccharine broth or agar containing brom cresol purple gives very clear indication as to acid production from the sugar present, unless the organism produces alkalinity from the peptone. This possibility should always be investigated by the use of some indicator with a more alkaline range, such as phenol red or cresol red. In case alkalinity is produced in the broth or agar without the sugar, it must be recognized that the production of acid may not raise the H-ion concentration of the medium above its original reaction. Theoretically the only way to be sure of acid production in such cases is to use some medium in which the organism grows well without producing alkalinity; but as such a medium is ordinarily difficult or impossible to obtain, this procedure is generally out of the question. The method recommended is to use a non-saccharine broth or agar as a check, both saccharine and non-saccharine media containing those indicators whose ranges cover the P_H changes likely to occur, and to pronounce the organism an acid-producer provided the H-ion concentration is greater in the presence of the sugar than it is in its absence. A very handy combination of indicators for this test is a mixture of brom cresol purple and cresol red. They may both be added to the same medium, giving a solution that changes very slowly from purple to yellow through a long range (from about $P_H = 8.0$ to about $P_H = 5.0$) extending to a considerable distance on both sides of neutrality. The exact amount of indicator used in the medium is not important as long as the color obtained is distinct. A convenient strength has been found to be 1 cc. of a 1.6 per cent alcoholic solution to the litre of medium. When brom cresol purple and cresol red are mixed, neither indicator need to be used in quite such great concentration.

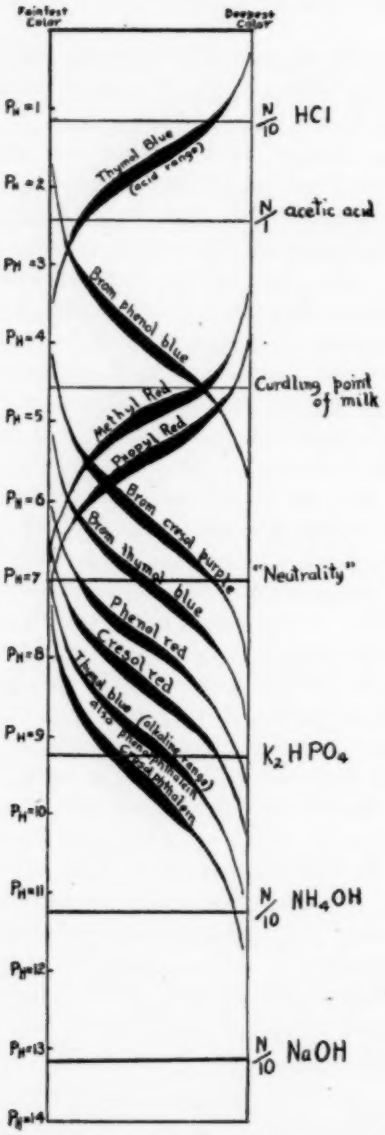

FIG. 1. COLOR CHANGES IN CLARK AND LUBS' INDICATORS. COMPARED WITH THE P_H VALUE OF CERTAIN IMPORTANT SOLUTIONS

Slightly modified from chart of Clark and Lubs

Relation of H-ion concentration to acidity

The above discussion will be readily intelligible to any reader who has an understanding of H-ion concentration and of the meaning of the symbol P_H. There would, indeed, be no point in discussing this subject further, if the research worker alone were concerned, because Clark and Lub's recent paper (1917) is readily available and treats the subject as fully and adequately as could be desired The new chart, however, is intended primarily for instruction and it is felt that teachers may desire a brief discussion of hydrogen-ion concentration for class-room or reference use. As there is at present no bacteriological textbook, so far as the committee know, that discusses the relation of acidity and reaction of media to H-ion concentration, and as copies of this report can be obtained from the secretary of the Society at about cost price, it seems well to summarize the subject here. Nothing new, however, is added in the following pages to what previous writers have said.

What is hydrogen-ion concentration?

The concentration of hydrogen-ions in a solution is used as a measure of acidity and alkalinity on the basis of the ionic theory. According to this theory, every salt, acid, or base, in aqueous solution, breaks up to some extent into "ions." Even pure water (H_2O) dissociates to a very small extent into H-ions and OH-ions. The H-ions bear a positive electric charge, the OH-ions a negative charge. Free H-ions are acid, free OH-ions are basic. In pure water both are present in equal amounts, thus neutralizing each other. Acids and bases dissociate to a much larger extent than pure water, the amount of dissociation depending ordinarily upon their strength. An acid dissociates into positive H-ions and negative ions consisting of the acid radicle. A base dissociates into positive metal ions and into negative hydroxyl (OH) ions.

The stronger acids (sulphuric and nitric, for instance) ionize to a greater extent than the weaker (acetic, for instance, and other organic acids). The more completely an acid ionizes in

solution, the greater the H-ion concentration of that solution. Therefore if different acids are dissolved in the same ratio of acid to water the H-ion concentration is an index of the strength of the acid. Similarly if different bases are dissolved in water in the same proportions, the OH-concentration is an index of the strength of the base.

If to a quantity of pure water (containing H and OH-ions) a drop of sulphuric acid be added, the mixture will contain H-ions, OH-ions and SO_4-ions. The number of free H-ions will be greatly increased because of the large number added in the drop of acid; but the number of hydroxyl ions will be decreased, because the change in ionic equilibrium will cause many of them to combine with free H- ons to form water. In other words, the H-ion concentration will be increased, the OH-ion concentration decreased. If, further, a larger quantity of the acid be added, the H-ion concentration will be still further increased, the OH-ion concentration still further decreased. It is evident, therefore, that H-ion increases and OH-ion concentration decreases as the acidity increases. Either may be used as an index of acid reaction; but H-ion concentration is preferred because it increases with increasing acidity.

When an acid is mixed with water containing other materials in solution, the matter is more complex. Various combinations are possible between the acid and the other materials in solution which prevent the H-ion concentration from being increased as much as it would have been if the same amount of acid had been added to pure water. Materials which thus repress the H-ion concentration of a solution are called "buffers." Organic matter in solution is especially likely to exert a buffer action. When buffers are present, the H-ion concentration of any solution will obviously be lower than it would have been with the same amount of acid added if no buffers were in solution. The actual reaction of such a solution is also lower, for only the ionized portion of an acid exhibits acid properties. The H-ion concentration, therefore, is a more correct index of the acid or alkaline reaction in a case like this than is the quantity of acid added.

When a base is added to water or to a neutral solution, the relation is exactly the reverse. The concentration of OH-ions is increased, that of H-ions is decreased. Either may be used as an index of alkalinity in terms of the ionic theory; but H-ion concentration is ordinarily used for the sake of uniformity. Hydrogen-ion concentration is inversely proportional to the dissociation of the base and therefore to the true alkaline reaction of the solution. Obviously a saturated solution of a strong base such as KOH has an exceedingly small H-ion concentration, but it is nevertheless measurable.

What is meant by P_H?

After deciding to use H-ion concentration as a measure of reaction the first necessity is to obtain some simple and concise method of expression. For this purpose the figures actually expressing H-ion concentration are quite unwieldy because of the extreme minuteness of H-ion concentration in weakly acid and in basic solutions. The H-ion concentration of pure water, for instance, has been shown to be 0.000,000,1 gram per litre, or in other words 0.000,000,1 normal (as a normal acid contains one gram of hydrogen per litre). A simpler method indicating this small quantity is by the logarithmic form of expression, i.e., 10^{-7} or log -7. This method of expression was adopted by Sörensen, who was the first to point out the great influence of H-ion concentration (as distinct from titrable acidity) upon biological activities. Sörensen used the symbol P_H^+ (or the simpler P_H) to represent this logarithm, without the minus sign; or using mathematical language, P_H is the logarithm of the reciprocal of the H-ion concentration expressed in grams per litre. Sörensen used this symbol to signify H-ion concentration. By this method of expression, the H-ion concentration of pure water is indicated by the formula $P_H = 7.0$.

Because P_H is an invert logarithm, it decreases with increasing H-ion concentration. Therefore, acid solutions have a P_H-value smaller than 7.0, basic solutions a P_H-value greater than 7.0. It has been found, for instance, that the P_H-value for $\frac{1}{10}$

normal HCl is 1.05, for normal acetic acid 2.4, while for $\frac{1}{10}$ molecular NH_4OH it is 11.2.

In interpreting P_H-values it must be remembered that adding 1 to the logarithm of a number is equivalent to multiplying the number itself by 10. For example, log 3 (i.e., 1000) = 10 × log 2 (i.e., 100). Similarly, to use an illustration that more nearly concerns us here, log −6 (i.e., 0.000,001) = 10 × log −7 (i.e., 0.000,000,1). In other words, a solution with a P_H-value of 6 has ten times the H-ion concentration of pure water ($P_H = 7$); and one with a P_H-value of 5 has ten times the H-ion concentration of a solution with a P_H-value of 6, or 100 times the H-ion concentration of pure water. Thus by a simple calculation it is possible to compute the relation between the H-ion concentrations of two solutions provided the difference in P_H-value is an integer. In case the difference in P_H-value is composed of an integer and a decimal, the relation may be learned by finding the number of which the decimal is the logarithm and giving it one more place to the left of the decimal point than there are units in the integer. An example will make this clearer:

For 1/10 normal HCl: $P_H = 1.05$
For normal acetic acid: $P_H = 2.4$

The difference is: 1.35
Now 0.35 = log 2.24,
But as the integer is 1, there must be two places to the left of the decimal point, i.e., 22.4 (approximately 22.5).
Hence tenth normal hydrochloric acid has approximately 22.5 times the H-ion concentration of normal acetic acid.

It is also possible to compute the OH-concentration for any solution of which the P_H-value is known, because there is a constant relation between the two expressed by the equation

$$P_H + P_{OH} = 14.$$

That is, P_{OH} may be determined for any solution by subtracting its P_H-value from 14. Thus for normal acetic acid ($P_H = 2.4$) the P_{OH} value is $14 - 2.4 = 11.6$.

Measurement of H-ion concentration

There are two different methods by which the H-ion concentration of a solution may be determined: the electrolytic method and the colorimetric method. The electrolytic method is generally the more accurate, and is applicable to a greater variety of solutions; but it is time-consuming and requires quite complicated apparatus. The colorimetric method is much simpler, is accurate enough for all bacteriological work and can be applied easily to any ordinary bacteriological culture medium.

The electrolytic method. The electrolytic measurement of H-ion concentration depends upon the fact that when a metal is in contact with an aqueous solution containing dissociated ions of that metal, there is an electromotive force set up between the metal and the solution, which varies according to the concentration of the ions of this metal in the solution. This electromotive force can be measured, and by means of the proper formula, the ionic concentration of this metal in the solution can be determined. Hydrogen acts like a metal in this respect when brought into electrolytic contact with an aqueous solution. This is accomplished by immersing a platinum electrode (platinum foil, coated with platinum black) in the solution and allowing a constant stream of hydrogen to bubble over the platinum. Because of the affinity of platinum for hydrogen, it takes up enough of the gas to act as a hydrogen electrode. The electromotive force established between this electrode and any aqueous solution with which it is in contact is inversely proportional to the H-ion exponent (i.e., P_H) of that solution.

The methods of measuring this electromotive force are quite complicated and are not adapted to the average bacteriological laboratory. All the average bacteriologist needs to know is that with the proper apparatus it can be measured and that the hydrogen ion exponent can be directly determined from it.

The colorimetric method. The colorimetric measurement of H-ion concentration depends upon color changes produced in certain substances (indicators) by varying acidity. Each indicator changes from one color to another between quite narrow limits of H-ion concentration, and between these two limits

every shade of the indicator corresponds to a definite P_H value. The best known indicators to the bacteriologist are litmus and phenolphthalein. Of these, litmus has little value in accurate H-ion measurements, partly because it is not a definite chemical compound, and partly because azolitmin, its chief component (even though chemically pure), varies so much in its color reactions according to the material present in the solution that little accuracy can be obtained with it. Phenolphthalein, on the other hand, gives very accurate results within the P_H-limits between which its color changes takes place; but this range (P_H = 8.0 to P_H = 9.6) is considerably to the alkaline side of neutrality. Bacteriological work, however, has special need of H-ion determinations near neutrality and in acid solutions.

In passing, it is of interest to notice that bacteriologists, in an empirical way, have realized for some time that the phenolphthalein color changes takes place in alkaline solutions. It has been found that bacteria grow better in solutions that are slightly acid to phenolphthalein, and it has lately become customary in this country to adjust media to 1 per cent normal acid to phenolphthalein. Bacteriological media have ordinarily contained Witte's peptone; and in 1 per cent solutions of this peptone, the above titre corresponds closely to the H-ion concentration of pure water (P_H = 7.0). This is not necessarily true of other peptones or of other media; but bacteriologists who have used peptone Witte have for some time adjusted their media practically to true neutrality.

The relation of different indicators to various points in the P_H-scale was studied by Sörensen as well as by others who have followed him. Most recently Clark and Lubs have furnished us (1917) with a beautiful series of accurate indicators whose sensitive ranges meet or even overlap and extend from P_H = 1 to P_H = 10. The relation of these indicators to H-ion concentration is shown in figure 1 and table 1. Of special interest to bacteriologists are the three indicators nearest to P_H = 7.0, i.e., brom cresol purple, brom thymol blue, and phenol red. Of these brom thymol blue is most useful in adjusting the reaction of media to neutrality, because it is yellow in acid solutions

and blue in basic solutions, passing through various stages of green between the points $P_H = 6.0$ and $P_H = 7.6$. At $P_H = 7.0$ it is grass-green, a shade that can be easily recognized by the eye after a little practice. As a result it is possible to obtain media that are essentially neutral, no matter what their composition may be, merely by adding sufficient acid or base so that brom thymol blue becomes grass-green when added to them.

It is plain that a rough idea can be obtained as to the H-ion concentration of any solution by simply finding which indicators give their acid color in it and which give their alkaline

TABLE 1

Color changes of Clark and Lub's indicators

INDICATOR	FULL ACID COLOR	FULL ALKALINE COLOR	SENSITIVE RANGE
Thymol blue (acid range)	Red	Yellow	1.2-2.8
Brom phenol blue	Yellow	Blue	3.0-4.6
Methyl red	Red	Yellow	4.4-6.0
Brom cresol purple	Yellow	Purple	5.2-6.8
Brom thymol blue	Yellow	Blue	6.0-7.6
Phenol red	Yellow	Red	6.8-8.4
Cresol red	Yellow	Red	7.2-8.8
Thymol blue (alkaline range)	Yellow	Blue	8.0-9.6
Phenol phthalein	Colorless	Red	8.0-9.6
Cresol phthalein	Colorless	Red	8.2-9.8

color. After a little experience with these indicators it is possible to do even better than this by inspection of the shade of color produced by whichever indicator or indicators are sensitive at the P_H-value of the solution in question. Accuracy, however, can be obtained only by actual comparison with the colors produced by the indicators in solutions of known H-ion concentration. For comparison Sörensen devised a series of standard solutions varying in H-ion concentration from $P_H = 1$ to $P_H = 13$. Clark and Lubs have proposed a different series of standards that are published in a more readily available place (loc. cit.). Of special interest is their standard corresponding to $P_H = 7.0$ (neutrality). This is prepared by mixing 5 cc. of $\frac{1}{5}$ molecular K_2HPO_4 with 3 cc. of $\frac{1}{5}$ molecular NaOH and dilut-

ing to 20 cc. with distilled water. Varying the proportion of these two solutions in the mixture increases or decreases the H-ion concentration as shown by table 1 of Clark and Lubs (1917, p. 26).

The difficulty of preparing such standard solutions of known H-ion concentration in the ordinary bacteriological laboratory has led Barnett and Chapman (1918) to devise a series of color standards that do not require accurate chemical adjustments. The standards that they propose are for phenol red, but they state that the method may be used for other indicators. Their method is to prepare two series of tubes, one series containing a

TABLE 2

Barnett and Chapman's color standards for H-ion determinations

Pair no. 1	.5 cc. acid + 9 drops* indicator;	5 cc. base + 1 drop indicator
Pair no. 2	.5 cc. acid + 8 drops indicator;	5 cc. base + 2 drops indicator
Pair no. 3	.5 cc. acid + 7 drops indicator;	5 cc. base + 3 drops indicator
Pair no. 4	.5 cc. acid + 6 drops indicator;	5 cc. base + 4 drops indicator
Pair no. 5	.5 cc. acid + 5 drops indicator;	5 cc. base + 5 drops indicator
Pair no. 6	.5 cc. acid + 4 drops indicator;	5 cc. base + 6 drops indicator

*In some laboratories it may prove simpler to use 10 cc. in each tube and to measure out the indicator solution in tenths of a cubic centimeter instead of in drops.

dilute acid solution, the other a dilute basic solution, adding decreasing quantities of the indicator to the acid series and increasing quantities to the alkaline series. The acid tube with the largest quantity of indicator and the alkaline tube with the smallest quantity of indicator form a pair (to be viewed together in transmitted light); the acid tube with the next largest quantity of indicator and the alkaline tube with the next smallest quantity form a second pair; and so on. This arrangement is shown in table 2.

It will be seen that the sum of the amount of indicator in the two tubes of each pair is always the same. Looking at the light through both tubes of any particular pair, the same color is obtained as when a solution of the proper H-ion concentration is viewed with an amount of indicator in it equal to the sum of

the quantity in the two tubes of the standard. The standard contains more indicator in the acid or in the alkaline tube respectively according to whether the pair of tubes is to represent the acid or the alkaline end of the range of the indicator.

Barnett and Chapman give the P_H-values corresponding to these successive pairs of tubes (using phenol red as indicator) as 6.9, 7.2. 7.5, 7.7, 7.9, and 8.1, respectively. Tested by a member of this committee they were found to correspond respectively to the P_H-values 7.1, 7.3, 7.5, 7.7, 7.8, and 7.9. Evidently the variation in the hands of different men is slight, probably less than in making up standard solutions of known H-ion concentration in the ordinary bacteriological laboratory. Such standards can be very quickly prepared even by a beginning student, and their use is therefore highly to be recommended.

The same method can be applied to brom thymol blue, thus covering a range more useful to some bacteriologists. The six pairs of brom thymol blue standards, prepared as given in table 2, correspond (as tested by a member of this committee) to the P_H-values 6.2, 6.4, 6.7, 6.9, 7.1, and 7.3, respectively. These two indicators together thus furnish standards covering the range from $P_H = 6.2$ to $P_H = 8.0$ with duplication at 7.1 and 7.3.

Why the titration method is illogical

The conventional method of adjusting the reaction of media or of determining the amount of acid produced by cultures depends upon titration to phenolphthalein. A computation is made of the per cent of normal alkali necessary to neutralize the solution, and the acidity is stated as equivalent to this per cent of normal acid. The assumption upon which this procedure is based is that the reaction of the solution is measured by the amount of base necessary to neutralize. This assumption is incorrect. Ten cc. of N/10 HCl and 10 cc. of N/10 acetic acid each require 10 cc. of N/10 NaOH to be neutralized, but the HCl is much more acid than the acetic acid.

In bacteriological media, moreover, there are nearly always present certain materials which have a buffer effect. Although

but weakly acid, they combine to a marked extent with the base used in titration and thus prevent the neutralization of the solution as rapidly as would be the case if no buffer were present. Peptone, for example, has a very strong buffer effect upon solutions in which it is present. As a result, in peptone solutions which are actually neutral, the titrable acidity may be quite high, because the NaOH used in titration combines with the peptone without greatly lowering the reaction of the solution. Hence titration is a very illogical method of determining the reaction of solutions containing peptone.

An example may make this clear. Witte's peptone is about neutral and its solutions are grass-green to brom thymol blue; but in a 1 per cent solution it titrates about 1 per cent normal acid to phenolphthalein. Difco peptone is more acid, giving a greenish yellow with brom thymol blue; but as it does not have such a marked buffer effect, it titrates lower than peptone Witte. As a result, now that Difco peptone is often used in America in the place of Witte peptone, it is not infrequent to find someone adding acid to it to bring it to 1 per cent normal to phenolphthalein, whereas alkali should be added to it to make it neutral to brom thymol blue. It should further be noticed that any peptone has practically the same H-ion concentration no matter what the concentration of the solution may be; but the titrable acidity is lower the more it is diluted. Hence the titration method leads to the error of adding acid to a 0.1 per cent solution of peptone to make its "reaction" the same as that of a 1 per cent solution.

A further fault of this titration method is that there is no sharp phenolphthalein neutral point. This indicator begins turning red at about $P_H = 8.0$ but does not reach its full alkaline color until about $P_H = 10.0$. Some bacteriologists titrate until its full color has appeared, others stop as soon as the first appearance of pink is evident, while still others try to use a point half way between these two limits. The result is considerable variation in the hands of different men.

All of these objections make the titration method entirely illogical for adjusting the reaction of media or for determining

the amount of acid produced by an organism. The colorimetric method of determining H-ion concentration is more logical, is of more significance, and is really more simple after the principles of the technic are once learned.

IV. THE REDUCTION OF NITRATES

The ordinary method of testing for nitrate reduction—as recommended in the last report of this committee (J. Bact., 3, 124)—is to grow the organism in a liquid nitrate medium and to test for nitrite after a definite length of incubation. The presence of gas is to be recorded if observed. On previous charts adopted by the Society the determination of ammonia was also called for, provision for making this determination having been made in the reports of the committees on water analysis of the American Public Health Association. It was distinctly stated in these reports, however, that ammonia may come from the peptone instead of from the nitrate, so that presence of ammonia in absence of nitrite does not necessarily indicate nitrate-reduction. This latter source of error, as has been pointed out by one member of this committee (Kligler, 1913), is greater than has been generally recognized by bacteriological writers. Hence no provision for the ammonia test is made on the latest chart recommended by the committee.

Omitting the ammonia test, however, opens up the possibility of error in another direction, for some organisms convert the nitrite into ammonia as rapidly as it is formed, so that its presence can scarcely be detected. This matter will shortly be further discussed by H. J. Conn and R. S. Breed in a paper (now in press) to appear shortly in this Journal. The nitrate-reduction test proves, indeed, to be a far more complicated matter than originally supposed. Absence of nitrite may mean any of the following things: (1) actual inability to reduce nitrate, (2) a medium so poorly adapted to the organism in question that growth is poor or lacking, (3) conversion of nitrite into ammonia as fast as produced, (4) assimilation of the nitrite-nitrogen (either as nitrite or as ammonia) as fast as

produced. Obviously a qualitative test for nitrite fails to distinguish between these four very different possibilities. A nitrite test accompanied by a quantitative ammonia test would undoubtedly prove an aid in detecting those organisms that reduce nitrate without an accumulation of nitrite; but as yet the committee has not worked out a quantitative test for ammonia simple enough for the bacteriological laboratory. Sometimes it is possible to obtain data of more significance by means of a qualitative test for ammonia, provided an ammonia-free medium is used containing no nitrogen other than the nitrate. In such a medium the only source of ammonia is the nitrate and its presence indicates nitrate-reduction even though nitrite be absent. As it is difficult, however, to obtain such a medium in which the majority of bacteria will grow, this method is realized to be of value only in special cases.

As a result the committee is unable at present to propose a standard method for the determination of nitrate-reduction. The provisional procedure recommended is as follows:

Inoculate first into nitrate broth and onto slants of nitrate agar. For this purpose use the standard formula for beef-extract broth (page 115 of 1917 report) with the addition of 0.1 per cent KNO_2, instead of the formula for nitrate broth given on p. 124 of the 1917 report. The richer medium is better adapted to pathogenic and to many other bacteria, and its greater viscosity aids in the detection of gas bubbles in case free nitrogen is produced. Test both the broth and the agar culture for nitrite by means of the reagents given on p. 124 of the 1917 report. (These reagents give the nitrite reaction as readily if poured on the surface of an agar slant as if added to a liquid culture). Presence of nitrite or of gas (indicated by foam in the broth or by cracks in the agar) shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary, as follows:

In case the fault seems to lie in poor growth, search for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increas-

ing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite or gas in any nitrate medium whatever should be recorded as nitrate-reduction.

If the organism grows well and yet produces no nitrite or gas, the determination must be recorded as doubtful unless the organism can grow well in some synthetic medium containing no nitrogen except nitrate. It is recommended that such an organism be tested in a medium containing small quantities of phosphate, calcium, chlorine, etc., with KNO_3 as a source of nitrogen and sucrose as a source of energy and of carbon.[3] Such a medium generally allows good growth with an organism capable of utilizing nitrate and sucrose. Unfortunately neither glucose nor lactose can be used in this medium as a source of carbon and energy, for the ordinary "c.p." preparations of these sugars contain much ammonia. If the organism in question grows (even but slightly) on a synthetic medium of this sort, it should be tested for nitrite by the usual method and for ammonia by means of Nessler's reagent (comparing with an uninoculated tube as a check). The presence of nitrite, of ammonia (i.e., a more pronounced ammonia reaction than in check tube), or of gas indicates nitrate-reduction.

The production of gas (free N) from nitrate is not a very common one; but a considerable number of soil organisms have this power, and one should be on the lookout for it in studying soil bacteria. The agar slant test is ordinarily a sufficiently delicate test; but, if liquid media are used, more reliable results may be obtained by the use of fermentation tubes.

REFERENCES

BARNETT, G. D., AND CHAPMAN, H. S. 1918 Colorimetric determination of reaction of bacteriologic mediums and other fluids. J. Amer. Med. Assn., **70**, 1062, 1063.

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[3] An illustration of such a medium which has proved satisfactory for some bacteria is: K_2HPO_4, 0.5 gram, $CaCl_2$, 0.5 gram, KNO_3, 1 gram, sucrose 10 grams, agar 12 grams, water 1000 cc.

BREED, R. S. 1914 The standard method of determining nitrate reduction. Paper read at Philadelphia meeting of the Society. Abstract in Sci., N. S., **41**, p. 661.

CLARK, W. M., AND LUBS, H. A. 1917 The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. J. Bact., **2**, 1–34, 109–136, 191–236.

CONN, H. W., ESTEN, W. M., AND STOCKING, W. A. 1906 Classification of dairy bacteria. Storrs (Connecticut) Agricultural Experiment Station, Rept. for 1906, 91–203.

CONN, H. J., ET AL. 1918 Methods of pure culture study. Preliminary report of the committee on the chart for identification of bacterial species. J. Bact., **3**, 115–128.

HARDING, H. A., AND PRUCHA, M. J. 1908 The bacterial flora of cheddar cheese. N. Y. Agr. Exp. Sta., Tech. Bul. 8.

HARDING, H. A. 1910 The constancy of certain physiological characteristics in the classification of bacteria. N. Y. Agr. Exp. Sta., Tech. Bul. 13.

KLIGLER, I. J. 1913 The source of ammonia and nitrates in a nitrate-peptone solution. Amer. J. Pub. Health, **3**, 788–792.

BACTERIAL VARIATIONS INDUCED BY CHANGES IN THE COMPOSITION OF CULTURE MEDIA

ANDREW IGNATIUS DAWSON

From the Department of Bacteriology, Columbia University, New York City

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INTRODUCTION

It is to be regretted that the tendency of investigation in bacteriology has been largely toward a study of effects, with little or no attempt to explain their fundamental causes. This is probably because the latter problems involve greater technical difficulties and offer less hope of immediate solution. Until a definite scientific foundation has been built up, however, on which to correlate the numberless facts already known, these will always remain isolated and to some extent unexplainable.

Bacteria, like all living cells, produce changes in internal composition as well as in external environment by means of chemical reactions. Living protoplasm, in order to maintain its viability, is constantly abstracting certain essentials from its environment, and replacing them by other substances, while it is, itself, in a constant state of alteration.

The changes wrought in the surrounding material by the presence of bacteria can only be definitely, that is, chemically, followed by cultivating them upon media of known and exactly reproducible composition, the so-called synthetic media. Suitable media of this type are not easily developed, but work is in progress along this line which cannot fail to bring to light many significant and fundamental relationships.

Changes within the bacterial bodies are even harder to follow. The present means which analytical chemists have at their disposal in the investigation of the most complicated of all known forms of matter, are palpably imperfect. Bacterial changes,

however, may be at times accompanied by (1) alterations in the empirical chemical composition of the whole bacterial bodies, and (2) alterations in the biological behavior of the organisms. Alterations in both these quanta are demonstrable, and can, at least in part, be correlated with their etiological factors.

In this paper we have attempted to add something to the data regarding the extent of alteration in the empirical composition of bacteria, to show how such alterations may be brought about, and to draw certain conclusions regarding their probable causes. We hope at least that the data here presented will be useful to future investigators in this field.

To produce a determinable change in either the properties or composition of bacteria, it is necessary to change their environment. This is easily done by first altering the nature of the medium upon which they are grown. By regulating the transplants with sufficient frequency to make sure that the changes observed are not due to bacterial "old age," and by continuing cultivation on the altered medium long enough to produce a reasonably complete adaptation of the organism to its new environment, the experiments can be adequately controlled, and an observable change of properties and composition, if such is possible, can be obtained.

The determination of the chemical composition of bacterial bodies is not an easy task. Ordinary bacterial growths are actually composed of very small quantities of material, and the production of sufficient bacterial substance for a chemical analysis is difficult.

PRELIMINARY PROCEDURE

In order to determine the biological change in bacteria in any direction, it is necessary (1) to preserve the same organism throughout all determinations, and (2) to determine the total activity of the organism in statu quo as far as possible, and (3) lastly, to subject the organism to constant varied conditions.

In accordance with these requirements, a strain of *B. coli*, which showed ready and abundant growth, was selected and preserved in all the following operations. It had been pre-

viously cultured on meat-extract agar for an unknown number of generations, of which at least 250 are known. In regard to this culture two questions present themselves: old age, and adaptation. If an organism is in a state of decrepitude, a transfer to another medium may mean only rejuvenation, which should assert itself in the next generation on any different medium on which the organism could have wide scope for its metabolic processes. A test of this possibility was made as follows: A twenty-four hour culture of the organism was emulsified in sterile salt solution of which a measured volume was plated on two different media: (1) meat-extract agar, and meat-infusion-glucose agar. After twenty-four hours' incubation the former showed 75 colonies, the latter showed 63. There seemed to be no distinguishing characteristic differentiating the organism from the colonies of the two plates. From this experiment the question of old age in this strain may be considered as eliminated.

The laws of adaptation, on the other hand, govern the internal state of the organism in its present conditions either fully or only partially. If the organism is fully habituated to its environment, a substantial change of environment effects a growth which is sparse; or, really or apparently abundant; or, no growth at all appears. If the abundant growth is apparent, and not real, it will be transitory; if real, it will persist.

Whether bacteria obey the laws of adaptation fully, partially, or not at all, may or may not be proven by external phenomena; but since the question should receive some answer from an examination of internal protoplasmic conditions, à priori an organism confined to one environment for many generations ought to throw light upon this point. The exact number of generations that will influence bacteria in this direction has not been determined. Arbitrarily, therefore, the 200th generation was chosen to initiate the solution of the question. In all the following experiments the organism had been confined to one medium for at least 200 generations before it was finally grown on this medium for examination.

In order to determine the biological activity of the organism in its present environment, it was cultured on a medium to which

it had been accustomed, i.e., agar, 2.5 per cent; peptone, 1 per cent; NaCl, 0.5 per cent; meat-extract, 1 per cent; incubated in test tubes 8 by 1 inch at 37°C. Under these conditions growth after twenty-four hours was fairly abundant, and even seemed to increase after that period.

To prove approximately an increase or decrease in growth after twenty-four hours' incubation, 100 tubes of different ages were selected, the planted area of which seemed to be of the same dimension. The growth was scraped from the medium at twenty-four hour intervals, transferred to weighing bottles

TABLE I

DAYS' GROWTH	APPROXIMATE WEIGHT OF BACTERIAL GROWTH IN 100 TUBES			APPROXIMATE WEIGHT OF BACTERIA AFTER DESICCATION			APPROX. AVERAGE PERCENTAGE LOSS BY DESICCATION
	I	II	III	I	II	III	
1	2.0	1.7	2.3	0.05	0.05	0.07	97
2	2.1	1.9	2.4	0.06	0.06	0.07	97
3	2.1	2.0	2.5	0.10	0.10	0.13	95
4	2.7	2.4	2.7	0.25	0.24	0.28	90
5	3.2	2.7	3.3	0.54	0.47	0.56	83
6	3.2	3.0	3.6	0.54	0.51	0.60	83
7	5.3	3.7	4.5	0.90	0.63	0.77	83
8	5.9	3.9	5.8	1.30	0.86	1.30	78
9	5.9	4.5	6.3	1.30	1.00	1.40	78
10	6.2	4.6	6.5	1.60	1.20	1.80	74
11	6.5	4.6	6.8	1.70	1.20	1.80	74
12	6.3	4.6	6.8	1.70	1.20	1.80	73
13	6.3	4.4	6.8	1.70	1.20	1.80	73
14	6.0	4.2	6.7	1.70	1.20	1.80	72

and weighed. After weighing, the material was carefully preserved at 0 to 5°C. for further examination. Growth of nine to eleven days proved to be the maximum.

In as much as the presence of moisture affects the weight of the collected bacteria, it is necessary to determine what relation moisture bears to the total weight. This determination will decide whether, and to what degree, the growth has been really or only apparently increased. Determinations of the water content were, therefore, made by drying the collected organism in weighing bottles at 97°C. over calcium chloride to constant

weight, which required seven days, and cooling the dried material in vacuo over the same dryer.

Experiments in this direction indicate that what holds true for bacterial derived substances,[1] also holds true for the whole bacterium, e.g., that bacteria show a great avidity for water. The water content of bacteria, therefore, seems to be subject to variations dependent on the period of incubation. The variation does not seem to depend on mere mechanical adhesion, because: First, of the difficulty attendant on desiccation; Second, of the power of absorbing moisture which dried bacteria or bacterial substance display to such an extent that three days' exposure to an ordinarily dry atmosphere increases the weight of the desiccated material 0.05 per cent.

It may not be contended that the entire loss of weight by desiccation is due to water alone, since bacteria may contain other volatile substances. The loss of volatile weight keeping pace with the age of the culture may be interpreted to mean that the organism in an environment of abundant food may need more than the ordinary supply of water, so that, the food decreasing, there is a decrease in the amount of water absorbed.

CULTURAL CHARACTERISTICS

Since the characteristics of the organism as grown on its accustomed medium are to be compared with those in a changed environment, a summary of the normal characteristics is necessary. In morphology, staining, and cultural traits, this organism showed only what is ordinary to *B. coli*. On Hiss' carbohydrate-serum-water medium, it produced acid and gas in glucose, levulose, galactose, maltose, sucrose, dextrin, and mannit.

BIOCHEMICAL CONSTITUENTS

Preliminary. The biochemical constituents are here understood as water and volatile matter; ash and ash constituents; sulphur; phosphorus; calcium; total nitrogen; amino-nitro-

[1] Leach, Journ. Biol. Chem., 1906, **1**, 463.

gen; fats and waxes; coagulable protein; soluble protein; carbohydrates; cellulose-like substances; residue.

In making determinations of this nature on bacteria, some of the well established methods are obviously inapplicable, and less well recognized ones had to be adopted, e.g., for extraction of fat. This necessitated, in such cases, before all else a careful investigation of the method.

DISCUSSION OF METHODS

Water and volatile matter were determined only by desiccation.

Ash. The material was incinerated in a silica crucible and kept at a dull red glow to constant weight; cooled in vacuo and weighed. *Constituents* of *ash* were subjected to ordinary qualitative and quantitative methods.

Sulphur. Total sulphur was estimated on both wet and dried bacteria. The material was heated with sodium carbonate and an excess of potassium nitrate, extracted with water, neutralized, and after precipitation weighed as barium sulphate.

Loosely combined sulphur was estimated as sulphide. The material was boiled four hours in 10 per cent KOH, neutralized with acetic acid, and precipitated as lead sulphide, care being taken during the process to prevent the formation of lead sulphate or carbonate.

Phosphorus. The material was first decomposed by boiling nitric acid, precipitated by ammonium-phospho-molybdate, redissolved in ammonium hydroxide, reprecipitated and estimated as magnesium pyro-phosphate.

Calcium. The material was decomposed by boiling sulphuric acid, precipitated by absolute alcohol, washed with 40 per cent alcohol at 34°C., and weighed as sulphate.

Total nitrogen. This was estimated by the macro-Kjeldahl method.

Amino-nitrogen was determined by the Van Slyke method.

Coagulable protein

Preliminary. Since a quantitative estimation of the protein content of the bacterial cell would assist in determining biological variations, the ideal method of protein extraction would involve the disintegration of the cell material without injury to the proteins. But the cellulose-like structure of the cell presents interference. To dissipate this, two alternatives are offered; to employ a cellulose enzyme, or to carry on extraction as far as possible by means of chemical reagents. Enzymes, however, are objectionable. Chemical reagents therefore must be resorted to, success depending on proper selection.

In exploring the effect of acids, alkalis, and neutral salts and solvents on proteins, it appeared that distilled water and sodium chloride possess greatest efficiency especially at reduced pressure. Of the two solvents, distilled water was discarded because it extracts fats together with proteins, and sodium chloride was selected as being a more powerful protein solvent.

The method of determining quantitatively the protein content of bacteria was therefore the following: Weighed amounts of wet bacteria were subjected to extraction by a definite amount of 6 per cent NaCl for twenty-four hours at 50°C. under 250 mm. pressure. After sedimentation, decantation of the supernatant liquid followed. The sediment was washed, and after the wash waters had been added to the original liquid, the whole was reduced to its original volume by evaporation. Part of this solution was weighed to ascertain the approximate protein content.

This solution heated to 85 to 90°C, with acetic acid to a concentration of 0.01 per cent, produces a coagulum, which when washed and dried on a weighed filter, is estimated as *coagulable protein*. The filtrate from the previous operation, after being diluted to 0.1 per cent NaCl was divided into two parts; one part was precipitated by HCl 0.1 per cent, and the other part by 0.1 per cent KOH. The precipitates were washed, dried, and weighed as *acid-precipitable* and *alkali-precipitable protein*.

The filtrates from the acid- and alkali-precipitations still gave evidence of containing protein not easily precipitated by mineral acids, alkalis, and neutral reagents. After neutralizing, the

protein content of these filtrates was estimated by Esbach's method, and listed as *soluble proteins.*

The insoluble residue containing a protein which resisted extraction, was subjected to tryptic digestion; the difference in weight before and after digestion was recorded as *insoluble protein.*

The substance remaining after the last operation, having given no protein reaction, nor showing the presence of nitrogen, was reserved for other examinations as *residue.*

Fats

Preliminary. Determinations of fat could not be made with accuracy until the known methods were examined. Experiments showed fat to be at least partly intracellular, and consequently total disintegration of the cell was necessary. The liberation of fat without its deterioration after cell disintegration waseffected by means of alkaline hypochlorite, commercially known as 'antiformin.'

Method. Weighed quantities of wet bacteria were treated with 20 per cent antiformin for twenty-four hours at 30°C., then warmed to 60°C. for one hour. After cooling to 15°C., and neutralizing with N/10 sulphuric acid, the solution was evaporated to dryness at 80°C., ground, and extracted with ethyl- or petrol-ether.

This residue was slowly boiled in 10 per cent NaOH, and after boiling, was rendered acid with dilute sulphuric acid. It was then diluted to three times the original volume with distilled water and cooled to 7° to 10°C., after which it was filtered. The precipitate was washed, dried, and extracted for five hours with petrol-ether.

Carbohydrates

Preliminary. When the bacterial residue, after protein extraction, is treated with concentrated sulphuric acid at ordinary temperature, it becomes turbid, and dark brown, finally turning

to clear deep red. If this reaction is hastened by gentle heat to 40 to 45°C. for one hour, diluted with 5 per cent KOH, and neutralized, the solution gives a reaction for reducing substances.

If the same process be carried on at a lower temperature, the activity of the acid is decreased, as is evidenced by the color, but the *amount* of reducing substance seems to increase. Also, whether normal bacteria or bacterial residue be employed in this process, it happens that when the reaction has proceeded to the production of a deep red coloration without turbidity, if cold distilled water be slowly added, a heavy gelatinous precipitate settles out of the solution. After neutralizing, this solution may be filtered and the precipitate recovered. The amount of reducing substances under these conditions is reported as *Carbohydrate* in terms of glucose.

The precipitate here obtained, after being washed by decantation, was found to yield a brown color with iodine, and not to be colored blue by iodine-zinc chloride. It is soluble in ammoniacal cupric oxide (Schweitzer's reagent), reprecipitated by acids, and is soluble in zinc chloride, from which it can be recovered on dilution with water. On hydrolysis with sulphuric acid reducing substances are formed.

These characteristics are essentially those of cellulose, and we regard the material so obtained as similar to, if not identical to that substance. The presence of cellulose in bacterial bodies has been a much discussed question, as throwing possible light on the vegetable nature of bacteria. The findings of Vaughan[2] seem to indicate that cellulose is absent in bacteria, but we are inclined to consider our results as confirming those of Dreyfuss, Hammerschlag, and others.[3]

The precipitate here obtained, and quantitatively estimated by collecting it on a weighed filter and weighing, was, therefore reported as *cellulose-like substance*.

[2] Prot. Split Prod., pp. 66-67.

[3] Zeitsch. fur physiol. Chem., 1887, **9**, 181.

DETERMINATIONS

After experimental examination, the above methods were adopted uniformly and without modification.

The material for these determinations was a nine days' growth of *B. coli*, collected at the stage of maximum development.

The media employed for obtaining growth, and representing change of environment, are described in the adjoined table 2.

Bacteria collected from these several media constitute the material for analyses by the methods already indicated, the number of determinations in each analysis varying from 7 for

TABLE II

Media employed in obtaining nine days' growth of B. coli for analysis

	SOLID MEDIA: VEHICLE, AGAR 2 PER CENT	TITRATION
I	Peptone, 1 per cent; meat extract, 1 per cent	Neutral
II	Peptone, 0.5 per cent; edestin 0.5 per cent	Alkaline
III	Peptone, 0.25 per cent, flour proteins, 1 per cent	Alkaline
IV	Peptone, 0.25 per cent, meat extract, 1 per cent, glucose, 1 per cent	Neutral
V	Peptone, 0.25 per cent, meat extract, 1 per cent, glucose, 1 per cent glycerol, 1 per cent	Neutral
VI	Peptone, 0.25 per cent, butter soap, 1 per cent	
VII	Peptone, 0.25 per cent, butter soap 1 per cent	
VIII	Peptone, none, potato juice, from whole unskinned potato, freed from starch, 500 grams potato to litre of medium	

ash to 20 for nitrogen. For the sake of brevity 3 of the most variant results are indicated in table 3.

Since table 3 does not include the results of all determinations, but only the most variant, for the purpose of comparison the average result for each series of determinations is therefore appended.

Table 4 presents variations in almost all bacterial constituents. Though strict interpretation of these results must necessarily await the light of further research, attention must be directed to the fact that from the simplest to the most complex constituents, variation is evident. The water content varies, which may not

TABLE III

Data pertaining to the analysis of B. coli grown on media I to VIII. Determinations and calculations are based on wet bacteria

	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water and volatile matter	74.45	72.52	60.28	75.10	75.00	60.70	60.36	79.62
	74.83	72.50	60.22	75.08	74.83	60.66	60.31	79.56
	74.86	72.43	60.20	75.00	74.96	60.60	60.26	79.50
Ash	4.80	2.73	2.53	4.51	4.50	7.84	7.69	2.14
	4.80	2.70	2.50	4.51	4.50	7.83	7.69	2.11
	4.80	2.69	2.44	4.49	4.50	7.83	7.67	2.03
Sulphur (total)	0.06	0.00	0.00	0.11	0.10	0.00	0.00	0.16
	0.05	0.00	0.00	0.09	0.10	0.00	0.00	0.14
	0.05	0.00	0.00	0.08	0.09	0.00	0.00	0.11
Sulphur (loosely combined)	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.03
	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.03
	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.02
Phosphorus (as P_2O_5)	4.25	3.50	2.38	2.90	3.31	1.71	1.83	0.92
	4.25	3.48	2.38	2.88	3.29	1.69	1.83	0.92
	4.22	3.47	2.37	2.87	3.28	1.69	1.83	0.92
Calcium (as CaO)	2.71	0.05	1.07	2.62	2.61	2.34	2.35	0.19
	2.67	0.05	1.06	2.62	2.60	2.33	2.33	0.18
	2.63	0.04	1.06	2.61	2.59	2.34	2.33	0.18
Nitrogen (total)	2.844	3.009	6.227	2.419	2.120	4.330	5.012	5.030
	2.842	3.012	6.222	2.410	2.113	4.330	5.009	5.026
	2.841	3.022	6.219	2.400	2.109	4.328	5.003	5.023
Nitrogen (amino-)	0.780	0.930	2.976	0.730	0.693	1.721	1.651	3.016
	0.777	0.887	2.969	0.721	0.690	1.689	1.650	3.011
	0.774	0.874	2.968	0.718	0.687	1.659	1.650	3.007
Protein, coagulable	2.99	1.00	4.70	2.35	2.50	4.10	5.35	5.57
	3.02	0.98	4.65	2.33	2.45	4.07	5.35	5.56
	2.98	0.98	4.65	2.31	2.44	4.03	5.31	5.53
Protein (acid-precipitable)	7.48	6.65	9.60	7.37	4.67	6.53	6.90	2.14
	7.45	6.62	9.56	7.30	4.58	6.49	7.01	2.09
	7.40	6.58	9.53	7.28	4.55	6.41	6.89	2.03

TABLE III—*Continued*

	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Protein (alkali-precipitable)	5.64	7.50	2.89	6.20	5.41	6.72	6.84	4.29
	5.61	7.47	2.83	6.01	5.32	6.63	6.79	4.24
	5.58	7.39	2.76	6.00	5.28	6.57	6.79	4.19
Protein (soluble)	0.06	2.20	1.47	0.09	0.14	0.09	1.22	1.14
	0.05	2.27	1.30	0.09	0.11	0.09	1.21	1.08
	0.04	3.01	1.22	0.05	0.08	0.08	1.21	1.07
Protein (insoluble)	0.05	0.05	0.10	0.07	1.01	0.08	0.13	0.04
	0.05	0.03	0.08	0.05	0.98	0.06	0.09	0.02
	0.05	0.03	0.08	0.03	0.98	0.06	0.09	0.02
Residue (insoluble)	1.03	2.40	2.05	1.43	1.76	1.00	1.52	0.81
	0.99	2.39	2.01	1.39	1.69	0.97	1.48	0.79
	0.99	2.32	2.00	1.39	1.63	0.95	1.43	0.75
Fats	4.06	4.40	4.91	5.80	8.01	*	*	5.10
	4.01	4.34	4.82	5.78	8.00			5.06
	3.98	4.29	4.78	5.75	8.00			5.05
Carbohydrate	4.03	3.13	2.22	1.41	2.73	3.06	2.95	1.04
	4.00	3.10	2.19	1.39	2.68	3.00	2.90	1.00
	3.97	3.09	2.17	1.35	2.66	2.97	2.86	0.97
Cellulose-like substance	1.03	2.45	2.06	1.46	1.79	1.10	1.55	0.90
	0.99	2.41	2.01	1.40	1.76	1.08	1.51	0.83
	0.98	2.39	1.97	1.37	1.70	1.07	1.49	0.76

*Not determined, because of extraneous fat attached to bacteria.

be as significant as other variations. There is an increase in the amount of ash and amino-nitrogen in the case of fat-media; protein is high where protein is present in the medium and highest where the medium-protein is in soluble form. Differences in quantity of coagulable protein show that the bacterial protein is, in many instances, itself different. This is supported by the fact that the ratio of nitrogen to any one of the protein fractions, or to all of them combined, is not constant. Also, it can be seen that the presence of certain sub-

TABLE 4

Summary of results obtained by use of different media in the cultivation of B. coli

	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Water and volatile matter	74.84	72.5	60.25	75.01	74.9	60.69	60.32	79.55
Ash	4.8	2.7	2.5	4.5	4.5	7.83	7.69	2.1
Sulphur (total)	0.06	0.0	0.0	0.1	0.09	0.0	0.0	0.14
Sulphur (loose)	0.0	0.0	0.0	0.02	0.01	0.0	0.0	0.03
Phosphorus P_2O_5	4.24	3.48	2.38	2.89	3.30	1.69	1.83	0.92
Calcium CaO	2.66	0.05	1.06	2.62	2.60	2.34	2.34	0.19
Nitrogen—Total	2.843	3.02	6.22	2.405	2.115	4.327	5.005	5.027
Nitrogen—Amino	0.771	0.891	2.970	0.724	0.691	1.696	1.650	3.012
Protein, coagulation	2.99	0.97	4.66	2.34	2.47	4.05	5.32	5.54
Protein, acid-precipitable	7.42	6.61	9.57	7.32	4.61	6.47	6.93	2.08
Protein, alkali-precipitable	5.60	7.44	2.80	6.05	5.33	6.63	6.80	4.22
Protein, soluble	0.05	2.25	1.31	0.07	0.10	0.08	1.21	1.10
Protein, insoluble	0.05	0.04	0.09	0.06	0.99	0.07	0.10	0.03
Residue, insoluble	1.00	2.37	2.00	1.40	1.70	0.98	1.49	0.76
Fats	3.99	4.32	4.82	5.77	8.00	*	*	5.07
Carbohydrate	4.00	3.10	2.19	1.38	2.69	3.01	2.88	1.00
Cellulose-like substance	1.00	2.42	2.01	1.41	1.75	1.09	1.52	0.81

*Not determined because of extraneous fat attached to bacteria.

stances in the environment, particularly fats, alters the chemical constitution of the organism.

BIOLOGICAL VARIATIONS

As the chemical constituents of the bacterial cell seem to be affected by a change in medium, so does it appear that the biological characteristics alter. Having seen that bacteria vary as stated above in regard to chemical composition when grown on different media, it seemed desirable to determine if such variation was accompanied (as might be reasonably expected) by variations in the biological activities of the organism.

From those biological characteristics that can be definitely measured, we have selected (1) the ability to produce enzymes capable of splitting different carbohydrates with the formation of gas and acid; and (2) the ability to agglutinate with immune rabbit serum prepared with the organism as antigen.

CARBOHYDRATES

The organism (*B. coli*) was grown on the 8 different media used above, and after about 200 generations was transplanted directly in every case to tubes of litmus-carbohydrate-serum-water, in which the production of acid and gas was noted. The time of the appearance of the phenomena in each case is recorded in table 5.

TABLE 5

Results of B. coli grown on 8 different media and transferred to litmus-carbohydrate-serum-water. "A" indicates hours required to produce marked acidity to litmus. "G" indicates hours required for first appearance of gas

CARBOHYDRATES	MEDIUM															
	I		II		III		IV		V		VI		VII		VIII	
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G
Lactose	24	24	24	24	18	18	10	10	10	10	10	10	10	10	48	48
Mannit	24	24	24	24	24	24	18	18	18	18	10	10	10	10	48	48
Sucrose	24	24	36	72	36	0	24	24	24	24	0	0	0	0	48	48
Maltose	24	24	18	18	18	18	18	18	18	18	18	18	10	10	36	36
Levulose	24	24	24	24	24	24	24	24	12	12	5	5	5	5	48	48
Glucose	24	24	24	24	24	24	24	24	24	24	5	5	5	5	36	36
Galactose	24	24	18	18	18	18	12	12	12	12	5	5	5	5	40	40
Dextrin	48	48	36	36	30	30	24	24	30	30	10	10	10	10	56	56

Since the organisms were originally the same, variations observed in the carbohydrate media are referred to changes of the bacteria during growth in the 8 media selected. From this table it is seen that great variations in the enzyme production of bacteria are produced by appropriate changes in the environment. This is evidenced by the widely differing results obtained by the organism after becoming accustomed to different metabolic conditions. The organism from medium I behaves precisely as a *B. coli-communior*, while from media VI and VII it possesses almost the type characteristics of a *B. coli-communis*. Although bacteriologists have been accustomed to regard them as distinct species, we have been able, by the aid of fatty acids in the culture medium, to obtain what practically amounts to a transposition from one to the other.

AGGLUTINABILITY

In order to test variability, if any, in the agglutinability of the organism after removal from the 8 specified environments, sera were prepared from rabbits injected with the organisms grown on media I, III, V, and VII. The sera were then used in producing agglutination of the organism grown on all the different media. The results are given in table 6.

TABLE 6

Agglutinating organism (B. coli) grown on

AGGLUTINATING ORGANISM AS ANTIGEN GROWN ON MEDIUM	MEDIUM							
	I	II	III	IV	V	VI	VII	VIII
I	400	400	400	400	400	400	400	400
III	500	500	800	600	700	400	400	700
V	450	450	630	700	850	700	750	500
VII	600	600	800	600	800	900	900	700

The fact that almost the same agglutination was observed in every case shows by the most conclusive test we know that serologically the organism retained the properties of the colon bacillus. The organism presents the most active antigenic properties when grown on a medium containing fat, the least when grown without protein. The maximum agglutination generally takes place when the antigenic and agglutinated organism are grown on the same medium.

This establishes a very definite proof of the constitutional change in the organism. The differences observed in agglutinability are easily as great as those frequently utilized to demonstrate the existence of different "strains" of the same basic organism.

Since antigenic differences appear to be produced so readily, it seems to us reasonable to suppose that they can be found in all bacteria, and can be referred to the immediate past environment of the organism: further real information they do not disclose. These findings warn us, on the other hand, that bac-

teria are capable of change: consequently, if the slightly variable strains, frequently kept for investigation in laboratories, remain on the same medium for a long period, they can be expected to lose their differential characteristics, exhibiting only those that are common to all the strains.

Connected with these fundamental changes we have sought evidence of morphological alteration. The organism grown on medium I showed a bacillus about 2 micra long, in thickness one-quarter its length; on medium III, generally 3 to 5 micra long, almost 1 micron thick; on medium VII, 3 to 5 micra long, more than 1 micron thick. The true significance of these morphological changes must be determined by the variations already noted.

GENERAL CONCLUSIONS

We have shown that by changing the character of the media upon which a given variety of bacteria is grown, the chemical constitution of the bacteria can be made to vary, and with it, certain biological characteristics such as agglutinability with immune serum and the formation of sugar-splitting enzymes. In some instances the variations practically amount to the "production" of a new strain. Accompanying morphological changes appear to be relatively unimportant.

THE VALUE OF A COOKED MEAT MEDIUM FOR ROUTINE AND SPECIAL BACTERIOLOGY[1]

W. L. HOLMAN

From the Pathological Laboratories, University of Pittsburgh, Pittsburgh, Pennsylvania

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The use of animal tissues added to media for the cultivation of bacteria is by no means new. The value of fresh sterile tissue added to various media has been clearly demonstrated for the growth of anaerobic microorganisms as in the method of Theobald Smith. The object of this paper is to bring to the attention of bacteriologists the cooked meat medium, now so widely used in the study of the anaerobic bacteria of war wounds, and to show its usefulness in routine bacteriological study as well as in more special lines of research.

Theobald Smith in 1890 first made use of unheated animal tissue for anaerobic growth. In 1905 Tarozzi, as Theobald Smith says, "rediscovered the method." He used 1 cc. pieces of tissue added to tubes of ordinary broth and found that he could heat the tubes to boiling for under five minutes and still get anaerobic growth. Tarozzi further showed that bouillon, made from infusion, heated, tubed without filtering and sterilized at 1040 to 105°C. for fifteen minutes, gave growth of obligatory anaerobes, while the filtered bouillon did not. The albuminous clot was decolorized during the sterilization and following the growth of the anaerobes became more or less red. In ten to fifteen days this unfiltered bouillon loses its ability to grow the anaerobes. If heated to 110° the medium is usually unfavorable. This author also found that by adding sterile tissues to ordinary bouillon and removing it at the end of some hours, the conditions for anaerobic growth were present in the bouillon.

[1] Read before the American Association of Pathologists and Bacteriologists, Philadelphia, 1918.

In 1899 von Hibler employed an emulsion of brain in water using open tubes and prolonged steam sterilization. He noted differences in the color changes resulting from the growth of various anaerobes and used the medium to divide these bacteria into two groups. One of these groups caused blackening of the brain emulsion and produced alkali while the other left the color unchanged and formed acid.

In his extensive study of the pathogenic anaerobes in 1908 von Hibler gave fuller details of the use of this brain medium. It is made of brain finely minced, mixed with from one-third to one-fourth its volume of water or normal salt solution, tubed in tall tubes and sterilized in flowing steam for one and a half hours. He had used this medium since 1894 and emphasized the advisability of boiling for half an hour just before seeding. He explained the anaerobic conditions as being due to slow reabsorption of oxygen owing to the consistency of the medium as well as to the presence of reducing substances in the brain tissue. The blackening which occurred from the growth of the proteolytic group he considered to be due to the action of sulphuretted hydrogen on iron in the presence of alkali. All the anaerobes von Hibler studied formed H_2S but the combination with iron only occurred where alkali was also produced. The use of muscle in place of brain was very briefly considered. The proteolytic group turned the muscle greyish or brownish-green to black while the active carbohydrate-fermenting group changed the color to red. Von Hibler further noted that the harmful effects of the acids formed in the meat on the bacteria were much less than those found in milk or the carbohydrate serum media.

Miss Robertson (1916) carefully analyzed von Hibler's results and substituted bullock's heart with equal quantities of water, for the brain in the emulsion, made the medium alkaline to litmus and found it equally useful for differentiation. She noted that the blackening may be delayed or absent if the medium remains acid.

Henry (1917) using the Robertson medium described further useful reactions for distinguishing certain of the anaerobes.

He pointed out that the muscle glycogen is converted into glucose and isomaltose so that the carbohydrate content in this medium approximates one per cent. The production of tyrosin in large amount by *B. histolyticus* in lesser quantities by *B. sporogenes* and most variably by *B. welchii*, are useful observations as indications of protein digestion. The red color change in the meat he considered due to an acid derivative of hemoglobin and according to the amounts of acid produced it is bright and stable or pink and readily decolorized. The blackening of the meat he believed to be due to the formation of iron sulphide but thought it might be a tyrosin derivative or a humin substance from condensation of an amino body with a sugar.

McIntosh (1917) made little or no use of the meat medium and the value of his important contribution to the classification of the anaerobic bacteria of war wounds suffers in consequence.

While working in the Robert Walton Goelet Research Laboratory in the Hospital Complementaire V.R. 76 at Ris-Orangis, and later at the American Red Cross Hospital of Paris in 1916–1917, I had experience with a cooked meat medium made from beef muscle in the study of the bacteria of war wounds. Since my return I have found it a most useful medium for the growth of a great variety of bacteria, anaerobic and aerobic.

The medium is made as follows: the fresh meat is freed from fat and gross fibers, finely minced, ground in a mortar and mixed with an equal part of water. It is then slowly heated to boiling with constant stirring to allow the soluble albumins to coagulate about the meat particles. This coagulated albumin serves in itself as a favorable medium for the anaerobes as Tarozzi has shown. The emulsion of meat is neutralized or made slightly alkaline, using hot titration with phenolphthalein, tubed at least two inches high and autoclaved at 115°C. for at least half an hour. Just before use, the tubes are put in flowing steam for half an hour and then rapidly cooled. The seeding is done by pipette, swab or needle and the material thoroughly mixed with the meat particles.

There are no further precautions necessary to obtain anaerobic conditions. All the anaerobes from war wounds which I have

studied grow readily in these open tubes such as *B. welchii*, vibrion septique, *B. oedematiens*, *B. tertius*, *B. fallax*, *B. aerofetidus*, *B. sporogenes*, *B. histolyticus*, *B. tetani*, *B. pseudotetani* and others. Not only do the anaerobes grow luxuriantly in this medium but the aerobes do equally well. In the routine study of war wounds I have found the cooked meat the best general medium for the growth of the mixed flora liable to be encountered. Hemolytic and non-hemolytic streptococci, capsulated cocci, staphylococci, Gram negative cocci, various diphtheroids, colon-like bacilli, members of the *B. mucosus-capsulatus* group, *B. proteus*, *B. pyocyaneus* and many other bacteria grow readily. The chain formation of the streptococci is particularly well shown and the morphological differences between the *Streptococcus viridans* and the *Streptococcus hemolyticus* groups are clear and have been confirmed by blood agar plating.

Perhaps the most favorable character of this medium, after its general growth stimulating influence, is that the products of growth do not rapidly destroy the various forms and of all the media in common use it is to the meat medium that one can constantly return to reisolate bacteria which have died out or have become hopelessly overgrown in other media. Just what the exact conditions are which favor this symbiosis is hard to say. The meat particles act no doubt as buffers to the extremes of reaction, especially absorbing undue amounts of the acids produced in the cultures. I have been able to reisolate a great variety of bacteria from the original mixed meat cultures which I brought with me from France. These reisolations from mixed cultures have been made after from six to ten months and it is quite remarkable the number of cases from which I have regrown the same types of organisms as were originally rapidly isolated. I have grown in this way streptococci of the viridans group, staphylococci, Gram negative cocci, diphtheroids and many others besides a great variety of anaerobes. Some of these latter were partially separated and were stored on the meat medium for further study. In no case, of those I have restudied, with two possible exceptions, have I failed to regrow the various anaerobes. The meat is the best single medium we have for

studying the anaerobes of war wounds, the reactions are useful for rapid differentiation of groups as well as for individual identification, and no other medium we have can so readily indicate the presence of anaerobes in mixed cultures where they are often not expected. I have confirmed the changes in the medium described and splendidly illustrated by Henry in my study of anaerobes from war wounds as well as for anaerobes from civilian cases and other sources. A fuller discussion of these will appear in a later paper on anaerobic bacteria.

I have also used this medium for growing other anaerobes with good results. A culture of *Actinomyces* which I isolated from a human case grows well in the open meat medium, in whitish granules which can be readily picked, examined in moist preparations or crushed and reinoculated in glucose agar shakes. The white granules of varying size develop best in a zone similar to that in a glucose agar shake. The growth so obtained makes useful material for student teaching in lieu of the fresh pus. Another anaerobe of interest here is the bacillus of acne which grows rapidly and particularly well in this medium the growth forming heavy white deposits on the meat particles in the upper portions of the tube. For these two anaerobes, which are more or less difficult to store, this medium is most useful. The culture of *Actinomyces* has been transferred after one hundred and fifty days and showed no evidence of dying out and the bacillus of acne is equally long lived and gives the typical ring growth in glucose agar on transfers up to at least five months. Cultures made from the scrapings of the teeth in this medium to indicate one of its many general uses, reveal growths of many leptothrix, fusiform and other microorganisms often seen in direct smear but rarely cultivated.

It is important to remember that in the use of fresh tissue, although this is still necessary for the growths of many of the stricter parasites, there is always present the disturbing possibility of contamination, not so much from without as from the animal tissue itself. This has been pointed out by many workers from Theobald Smith (1890) to the later work of Ford, Tarozzi, Adami, Wolbach and Saiki and many others. It is so uncertain

a factor that fresh tissue in culture media cannot be employed as a method for the routine work for the general bacteriological laboratory and it is just here that the cooked meat medium fills a long felt want.

De Kruif, Adams and Ireland in confirming the results of Bull and Pritchett on the toxin production of *B. welchii* found the amount of toxin to be as great in a veal mash broth as with fresh sterile tissue.

This meat medium finds in the study of the anaerobes perhaps its most useful place and offers a broad field of study for the biological chemist as well as for the bacteriologist. Investigation of the results of proteolysis by pure cultures of well identified anaerobes has received but little attention. The study of crystal forms which moist preparations show to be abundant in many of these cultures will undoubtedly amply repay the time expended. The application of these studies to the activities of the anaerobes in the intestinal tract, more especially in putrefactive conditions, may well lead to a clarification of many vexed questions.

CONCLUSIONS

The cooked meat medium is the most useful medium we have at present for obtaining growth of both anaerobic and aerobic bacteria, for storing mixed cultures for later isolation as well as pure cultures for further investigations.

We have in this medium a ready means for the cultivation of the anaerobes and the storing of pure cultures. The changes in reaction, color, odor and the evidences of varying degrees of proteolysis make it of the greatest importance in differentiating many of the types and adds to its value. It is further most useful in growing and storing cultures of the *Actinomyces hominis*, *Bacillus acne* and many others.

It is a medium simply made which can be readily sterilized and owing to its high buffer character is adaptable to the growth of a great variety of bacteria.

REFERENCES

Bull and Pritchett 1917 Jour. Exper. Med., **26**, 119.
De Kruif, Adams and Ireland 1917 Jour. Infect. Dis. **21**, 580.
Ford 1900 Trans, Assoc. Amer. Phys., **15**, 389.
Henry 1917 Jour. Path. and Bact., **21**, 344.
McIntosh 1917 Report No. 12 Med. Res. Committee, Great Britain.
Robertson 1916 Jour. Path. and Bact., **20**, 327.
Smith, Theobald 1890 Cent. fur Bakt., **7**, 502.
Smith, Brown and Walker 1905 Jour. Med. Res., **14**, 193.
Tarozzi 1905 Cent. fur Bakt. Abt. i (Orig.), **38**, 619.
von Hibler 1899 Cent. fur Bakt., **25**, 513.
von Hibler 1908 Untersuchungen uber die Pathogenen Anaeroben, Fischer, Jena.
Wolbach and Saiki 1909 Jour. Med. Res., **21**, 267.

THE DELAYED DEVELOPMENT OF COLONIES ON PLATES SEEDED FROM DISINFECTED SEWAGE

W. S. STURGES, JR.

Bacteriologist, Sewage Experiment Station, New Haven, Connecticut

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An experiment station for the study of various methods of treating the sewage of New Haven was operated by the city during the year June 1917 to May 1918, under the direction of a Committee of which Prof. C.-E. A. Winslow of Yale University was Chairman; and the author desires to express to Professor Winslow, and to Prof. L. F. Rettger, his thanks for advice and suggestions in regard to the work reported here. Chemical analyses were made by Dr. F. W. Mohlman, Chemist in charge of the station.

The New Haven Sewage Experiment Station offered peculiar opportunities for observing the effect of three widely used disinfectants—namely, copper, chlorine and sulphurous acid—on the bacterial content of sewage. The first of these substances occurs in the sewage itself in varying amounts during the working hours of the day, being derived from a trade waste discharged into the sewage by munition works at a distance of $2\frac{1}{2}$ miles above the station. During the manufacturing process in question shells are washed in hot water and sulphuric acid. This acid wash containing considerable copper sulphate is discharged into the sewer. The alkalinity of the sewage is sufficient to neutralize this acid waste except in occasional instances. The copper sulphate is probably changed first into the basic carbonate and then gradually converted into the hydroxide. Since the latter is insoluble it is presumably before this last change takes place that the copper exerts its disinfecting action. The other disinfectants discussed, sulphurous acid and chlorine, were added to the sewage as a part of our investigation. The sulphur

dioxide was applied as part of the Miles acid process of treatment in quantities sufficient to keep the effluent faintly acid. The acidified sewage was retained for four hours at the end of which time bacteriological examination was made. Liquid chlorine was added to the effluents of three of the processes under investigation in the usual way, plates being made after a period of contact of thirty minutes.

ANTISEPTIC ACTION OF COPPER SALTS

It was soon apparent, after beginning our investigation, that trade wastes were exerting a strong disinfectant action on the sewage at this outlet. A comparison of the counts made at different hours of the day indicates this fact.

It will be seen from tables 1 and 2 that the early morning and Sunday samples of sewage have very much higher counts than samples taken at hours when factory wastes might be expected to be present.

Evidence as to the exact source of the disinfectant was sought by taking samples at different points along the sewer from its outfall upward along its course. The results are given in table 3.

It will be noted that above the munition works a total count of nearly 1,000,000 bacteria per cubic centimeter and 100,000 gas formers per cubic centimeter was found, while below the munition works the total count was reduced to a few thousand and the gas formers to 100. Above the munition works no copper was detectable while the sewage immediately below contained nearly 9 parts per million. All samples were alkaline. It seems reasonable, therefore, to conclude that the low count of the sewage noticed throughout the week days in our investigation was due to the disinfectant action of this copper waste. This is quite in keeping with the results obtained by Johnson and Copeland[1] on the application of copper as a disinfecting agent for sewage. Some of their results at the Columbus Testing Station are shown below.

[1] Journ. Infect. Dis., Sup. I, 327, 1905.

TABLE 1

Disinfection of sewage by copper, August 10, 1917

TIME	PARTS PER MILLION				DECOLORIZATION TIME METHYLENE BLUE		BACTERIA PER CUBIC CENTIMETER 20 °C.	GAS FORMERS PER CUBIC CENTIMETER 37 °C.
	Chlorine	Alkalinity	Iron	Copper	Undiluted	1 2 Sewage water		
a.m.					hours	hours		
6.00	30	94	0.1	0.56	6	120	3,700,000	100,000
7.00	3,750	136	0.2	1.52	6	24		
8.00	3,100	160	5.0	1.36	24	72	2,500,000	1,000,000
9.00	2,900	101	1.8	2.88	48	120		
10.00	2,800	43	0.3	4.80	168	192	19,000	100
11.00	2,750	114	0.4	2.72	60	144		
12.00	2,800	98	0.2	4.00	144	168	559,000	1,000
p.m.								
1.00	3,075	6	5.5	5.60	240	240		
2.00	3,200	86	0.6	1.92	24	72	187,000	10,000
3.00	3,325	91	0.7	1.60	24	120		
4.00	3,375	88	0.2	1.92	48	96	39,000	100
5.00	3,450	98	0.1	3.52	150	144		
6.00	130	103	0.4	2.88	216	100	45,000	1,000
7.00	95	63	1.4	1.28	72	80		
8.00	52	92	0.2	0.64	24	96	33,000	1,000
9.00	50	96	0.0	0.44	6	24		

TABLE 2

Bacterial content of sewage, July 9–26

DAY	TOTAL COUNT PER CUBIC CENTIMETER, AGAR 20°C.				GAS FORMERS PER CUBIC CENTIMETER, 37°C.			
	8.00 a.m.	10.00 a.m.	1.00 p.m	4.00 p.m.	8.00 a.m.	10.00 a.m.	1.00 p.m.	4.00 p.m.
Monday...	1,500,000	225,000	59,000	16,000	100,000	1,000	1,000	100
Tuesday..	1,080,000	128,000	5,000	7,500	100,000	100	1,000	100
Wednesday.....	1,670,000	81,000	7,000	100,000	100,000	100	100	1,000
Thursday.	1,100,000	148,000	1,500	12,000	100,000	10,000	100	10
Friday....	1,125,000	42,000	9,000	9,000	100,000	100	100	100
Saturday.		68,500	7,100	800,000*		100	10	100,000*
Average.....	1,295,000	115,400	14,700	159,000	100,000	1,900	385	17,000
Sunday...		3,355,000	2,275,000	2,535,000		100,000	100,000	100,000

*Factories shut down at noon Saturday.

The period of contact in our case is the length of time that it takes the sewage to flow from the munition works to our laboratory. This we compute to be in the neighborhood of one hour. Our analysis (table 3) showed 8.8 parts of copper per million at the munition works but this is doubtless often exceeded, as we frequently obtained samples with lower bacterial content. It is to be expected therefore that the bacterial content of our sewage might frequently be reduced to a few thousand per cubic centimeter.

TABLE 3

Source of copper in the New Haven sewage

DESCRIPTION OF SAMPLE	NUMBER	PARTS PER MILLION		BACTERIA PER CUBIC CENTIMETER 20°C.	GAS FORMERS PER CUBIC CENTIMETERS 37°C.
		Alkalinity	Copper		
Near source of sewer	1	54	0.0	990,000	100,000
Below munitions factory	2	90	8.8	3,000	100
	3	165	5.6	3,000	1,000
Small branch sewer	4	134	0.0	559,000	10,000
Main sewer	5			70,000	100
	6	156	1.9	380,000	10,000
	7	90	3.5	65,000	1,000
Experiment station	8	130		53,000	1,000

On noting some of the very low counts obtained it occurred to us that possibly we were not finding all of the organisms that were really present. This proved to be the case. By prolonged incubation a count often developed many times greater than the one obtained at the end of two or three days. This phenomenon was so marked in the case of the "disinfected" samples that it was suspected that they differed in this respect from the samples containing no trade waste.

Accordingly in making counts of subsequent samples of raw sewage plates were kept in the incubator and recounted at the end of seven days. The "disinfected" samples as a rule show a marked increase on prolonged incubation. This increase is

quite variable as doubtless is the amount of copper in each sample. Frequently however the count at the end of seven days shows an increase of several hundred per cent over the three day count. This increase tends to be most marked in the samples having low initial count, that is in samples that have been most completely disinfected.

Space does not permit the full tabulation of all the results obtained. Seventy samples of raw sewage which were collected on Sundays or Saturday afternoons and at 8.00 a.m. on week-days (sewage containing relatively small amounts of copper wastes) gave an average of 222,000 bacteria per cubic centimeter after three days' incubation and an average of 348,000 after seven days' incubation. The per cent increase for individual samples between three and seven days varied from 2 per cent to 478 per cent and averaged 93 per cent. On the other hand 68 samples of sewage collected on week days after 8.00 a.m. (containing large amounts of copper wastes) gave an average count of 85,000 per cubic centimeter after three days and an average count of 155,000 per cubic centimeter after seven days. The per cent increase from the third to the seventh day varied from 17 to 1000 per cent and averaged 203 per cent.

A closer measure of the effect of the copper salts in delaying the development of colonies among the bacteria which they fail to kill outright is obtained by grading the samples of crude sewage according to their actual copper content. This was roughly accomplished as follows.

In determining the non volatile suspended matter in our chemical analyses we ignited the mat in a Gooch crucible after filtering 100 cc. of the sewage. After ignition the residual suspended matter on these mats was often found to be distinctly red, owing no doubt to the presence of cupric oxide. Each sample was placed in one or another of five groups according to the depth of color in the ignited mat. This crude determination probably gives a rough indication of the amount of copper salts present. In table 5 the samples of specially high and specially low copper content are compared upon this basis.

The count for the low copper samples doubled on incubating

for seven days while the seven day count for the samples high in copper was over four times as great as the three day count. The average increase on prolonged incubation was three and one half times greater in the high copper than in the low copper group.

Three explanations of this delayed colony formation on the plates seeded with sewage containing copper salts suggest themselves. In some instances it might be assumed that the copper was present in sufficient strength to exert an inhibitory action in the plates after the diluted sewage has been mixed with the

TABLE 4

Effect of copper sulphate on bacteria in Columbus sewage (Johnson and Copeland, 1904). Composition of sewage

ORGANIC NITROGEN	ALKALINITY	BACTERIA PER CUBIC CENTIMETER
p.p.m.	p.p.m.	
6.3	376	1,200,000

Cu	TOTAL COUNT ON GELATIN AT 20°C.			
	Contact: 0	1 hour	6 hours	24 hours
p.p.m.				
0	1,200,000	1,200,000	6,000,000	14,000,000
5	1,200,000	14,000	700	3,400,000
25	1,200,000	9,500	250	200
250	1,200,000	3,000	190	35

media; although the antiseptic must here be in a very high dilution. If the original sewage sample contained 10 parts per million a plate poured for a dilution of 1:100 would contain about 1 part of copper in 10,000,000 parts of medium. Still it is conceivable that even such a small amount of a strong antiseptic might retard the growth of some of the organisms we are dealing with when they are suddenly placed in such a new environment. A single, isolated organism, especially if of animal origin, might well be retarded a few days by an extremely slight amount of an inhibiting substance. An attempt was made to confirm this view by adding slight amounts of a copper salt solution to a sample of Sunday sewage at the time of plating.

TABLE 5

Three and seven day counts of raw sewage containing various amounts of copper

LOW COPPER CONTENT—BACTERIA PER CUBIC CENTIMETER			HIGH COPPER CONTENT—BACTERIA PER CUBIC CENTIMETER		
3-Day count	7-Day count	Percentage increase	3-Day count	7-Day count	Percentage increase
144,000	269,000	87	245,000	395,000	20
120,000	244,000	104	23,000	98,000	330
160,000	312,000	95	9,000	53,000	490
132,000	277,000	110	33,000	73,000	121
126,000	270,000	114	41,000	150,000	266
134,000	396,000	200	127,000	252,000	99
142,000	340,000	140	7,000	43,000	510
245,000	395,000	61	3,000	15,000	400
300,000	465,000	55	10,000	47,000	370
70,000	175,000	150	185,000	320,000	73
36,000	117,000	225	3,000	12,000	300
65,000	135,000	110	3,000	33,000	1000
230,000	500,000	115	3,000	20,000	566
34,000	196,000	480			
115,000	170,000	48			
110,000	360,000	240			
168,000	285,000	70			
375,000	660,000	76			
250,000	530,000	112			
290,000	420,000	50			
154,000	183,000	18			
7,000	7,500	7			
150,000	355,000	136			
21,000	29,000	40			
177,000	292,000	65			
50,000	60,000	20			
152,000	245,000	62			
190,000	255,000	34			
98,000	228,000	133			
23,000	28,000	22			
205,000	340,000	66			
154,000	249,000	63			
250,000	445,000	78			
165,000	280,000	70			
Average increase for low copper samples		101	Average increase for high copper samples		349

Counts were made at the end of three and seven days incubation but neither 5, 10 nor 20 parts of copper caused any appreciable increase in the difference between the first and second count.

The second explanation that suggests itself is that prior to plating some of the organisms have been injured, so to speak, by the copper so that it takes them a few days to recover. They are not killed but are in such a weakened condition that immediate growth is impossible. A purely hypothetical explanation of this lag period is that it is necessary for the previously absorbed antiseptic to diffuse out of the organisms before growth will take place.

A third possibility is that the organisms which survive the copper treatment may be of slowly growing types. We thought at first that the presence of a large proportion of spore formers might account for the phenomenon but a few heating tests failed to show a specially high proportion of spore bearing organisms on the seven day plates.

ANTISEPTIC ACTION OF SULPHUROUS ACID AND SULPHITES

In the Miles' acid process of sewage treatment we added sulphur dioxide in sufficient quantities to neutralize the alkalinity of the sewage and keep the effluent constantly acid. The sewage with a free acidity of 50 to 100 parts per million (expressed as calcium carbonate) was retained in a sedimentation tank for four hours. Plates of the effluent were then poured and counts made after three and seven days incubation. As may be seen from table 6 a very marked lag period was noted on the plates seeded with this effluent.

While a two or three day count is usually remarkably low this is therefore by no means a correct measure of the number of living organisms present in the Miles' effluent. Prolonged incubation will often indicate a total count of ten times this magnitude. The average of the percentage increase of the total counts between three and seven days incubation is 560 per cent. Such delayed growth should be taken into consideration in any bac-

teriological examination of this type of sewage effluent. It is easy to imagine that the bacterial removal is greater than it really is. On the other hand the bacterial efficiency of the

TABLE 6

Three and seven day counts of Miles' acid effluents showing the delayed development due to sulphurous acid

3-DAY COUNT	7-DAY COUNT	PERCENTAGE INCREASE	3-DAY COUNT	7-DAY COUNT	PERCENTAGE INCREASE
620	2,290	269	22,000	71,000	220
8,000	22,200	178	2,570	9,000	250
700	1,510	116	1,100	3,300	200
900	7,600	744	4,850	13,000	170
5,400	12,000	122	200	1,900	850
6,400	20,000	212	200	2,800	1300
1,240	9,200	642	80	190	140
31,000	42,000	35	245	3,200	1200
15,000	40,000	166	1,000	4,700	370
2,500	19,300	672	2,000	7,000	250
1,700	7,000	316	200	1,000	400
590	8,000	1,250	30	90	200
2,150	11,000	411	30	60	100
42,800	110,000	157	150	1,400	830
32,000	100,000	210	40	360	800
268	1,800	570	10	600	5900
1,140	4,185	270	1,800	11,000	510
3,800	14,400	280	41	96	130
2,760	9,500	240	50	1,600	3100
430	1,100	160	35	350	900
3,200	8,300	160	105	805	670
1,000	9,000	800	400	1,100	170
460	3,920	750	233	1,600	590
3,800	14,400	280	600	1,600	170
20	140	600	130	390	200
570	3,350	490	1,140	3,200	180
20,000	44,000	120	470	2,580	450
2,100	7,100	240	360	1,500	320
6,500	20,000	210	535	4,400	720
860	4,500	420	225	3,300	1370

Average Increase, 560 per cent

Miles process is so high that even the number of colonies obtained after seven days incubation is but small and there may be no sanitary significance to the organisms that develop after

three days incubation. It seems probable from our low *B. coli* counts that the pathogens are practically eliminated. It was not possible in our work to do anything in the way of classifying these organisms showing delayed growth except to find, as noted above, that they were mostly non-spore formers.

To find the number of organisms per cubic centimeter in this low count effluent it is necessary to pour plates with a dilution of only 1 to 10. In this low dilution the free sulphurous acid might be present to the extent of one part per million of media, besides which there are considerable amounts of sulphites present. This is doubtless sufficient to exert an antiseptic action in the plates during incubation. Some better method of getting a total count is highly desirable. Trials were made in which the effluent was neutralized before plating; but the antiseptic action was still very strong, probably due to the presence of sulphites.

ANTISEPTIC ACTION OF CHLORINE

Finally, in connection with our study of the effect of varying amounts of chlorine on the bacterial content of raw sewage, plates were poured immediately before chlorinating and after being exposed to chlorine for one half hour. The plates of some of these chlorinated samples, with those of the corresponding raw samples, were incubated for seven days and the count then compared with the three day count. 6 and 8 parts per million of liquid chlorine were employed. The results are shown in table 7.

The results of this limited number of observations appear rather erratic. It is evident, however, that there is no increase in the seven day over the three day count to compare with that in the case of samples disinfected by sulphurous acid. In general there is no very marked difference between the chlorinated and the corresponding raw samples. In 16 out of the 24 cases there was a slightly greater increase in the case of the chlorinated samples while in the other 8 cases the increase was actually less in the case of the chlorinated samples. If we take the averages we find that the increase for the chlorinated is about

the same as that for the unchlorinated samples. If we were justified in drawing any conclusion from this table it would be that chlorine when used in amounts sufficient to have a very

TABLE 7

Three and seven day counts of chlorinated and unchlorinated samples of raw sewage

CHLORINATED			CORRESPONDING RAW		
3-Day	7-Day	Percentage increase	3-Day	7-Day	Percentage increase
16,600	23,500	42	380,000	384,000	1
500	500	0	9,600	18,000	88
3,000	12,000	300	138,000	162,000	17
3,340	7,000	110	55,000	80,000	45
1,000	3,200	220	22,000	116,000	430
1,300	2,300	77	224,000	340,000	52
8,000	18,000	13	18,000	38,000	111
16,000	24,000	50	175,000	243,000	39
1,250	3,200	156	66,000	95,000	44
4,000	10,000	150	127,000	252,000	98
2,000	14,000	600	157,000	287,000	83
2,600	6,400	146	390,000	800,000	105
700	1,300	86	10,000	28,000	180
1,350	2,650	96	4,000	15,000	275
90,000	146,000	62	380,000	605,000	59
106,000	180,000	70	720,000	1,060,000	47
3,000	4,400	47	320,000	340,000	6
580	710	22	17,000	31,500	85
1,500	1,600	7	141,000	225,000	60
820	2,100	156	160,000	300,000	88
1,900	3,500	84	119,000	165,000	39
2,800	4,400	57	82,000	120,000	46
1,000	3,500	250	4,900	16,000	227
125	125	0	15,000	41,000	173
Average increase chlorinated		115	Average increase unchlorinated		100

strong bactericidal action (90 per cent removal or better) has little or no tendency to cause delayed colony formation among the surviving organisms.

SUMMARY

If copper salts are added to sewage in sufficient quantities to effect considerable bacterial reduction there is a marked tendency to delayed colony formation among the surviving organisms.

In previous investigations such as that of Johnson and Copeland some of the "disinfectant" action observed was doubtless merely due to this property.

Sulphur dioxide when used to disinfect sewage has an even greater tendency to produce delayed colony formation than have copper salts.

This phenomenon interferes with the common method of determining the count. The actual bacterial content of a Miles' acid effluent may readily be ten times as great as a two or three day 20° count.

Such evidence as has been obtained suggests that chlorine does not produce delayed colony formation of the type indicated.

The delay in colony formation is due either:

a. To the antiseptic action of minute amounts of copper or sulphurous acid or sulphites carried over in the dilution process into the plates. This action is probably important in the case of the sulphurous acids and bisulphites where plates were made at only a 1 to 10 dilution. It is probably relatively slight in the case of the copper with plates poured after 1 to 100 dilution and containing only 1 part of copper in 10,000,000 parts of medium.

b. To a selective action of the disinfectants which may perhaps kill out quickly growing and spare slowly growing types. As throwing light upon this possibility it should be noted that no evidence was found of an increased proportion of spore bearers among the slowly growing forms.

c. To a possible lowering of the vitality of the organisms affected by the disinfectant so that while not killed outright they are able to develop only slowly and after an appreciable lag period when transplanted to a favorable medium.

The completion of the operating period planned for the experiment station prevented a further study of these various possi-

bilities. The results so far obtained are presented here in view of their practical bearing on the period of incubation to be adopted for plates seeded with certain types of disinfected effluents and because of the theoretical interest of the possibility that the slow development of colonies on plates made from such effluents is due to a lowering of vitality produced by a sublethal dose of disinfectant.

METHODS FOR THE ISOLATION AND CULTIVATION OF BACILLUS PUTRIFICUS AND OTHER OBLIGATE ANAEROBES

WM. S. STURGES, JR. AND LEO F. RETTGER

From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University

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Bacillus putrificus is an almost ubiquitous putrefactive anaerobe which has received comparatively little study. Its important rôle in the natural decomposition of protein material was discovered by Bienstock (1899 and 1901) who has given us a graphic description of this, as yet more or less obscure, organism. Bienstock's observations were in the main substantiated by Rettger (1906, 1908). On account of its somewhat close resemblance to *B. tetani*, particularly in its morphology and certain cultural requirements, *B. putrificus* has often been mistaken for the tetanus bacillus. From this standpoint alone a wider knowledge of the important properties of *B. putrificus* seems desirable.

Although the Bienstock bacillus is so widely distributed in nature, it soon becomes apparent to any one who attempts to isolate it from its natural sources that this process is extremely difficult. Repeated efforts in this laboratory to effect a complete isolation by methods heretofore in vogue for anaerobes have always failed. Colonies seldom develop in the Novy jar, and when they do develop they are found to be impure. The tendency of this organism to grow into colony form only when it is associated with some other organism which favors its development is very marked, whereas *B. oedematis* and *B. chauvei* (*anthracis-symptomatici*), its close allies in the processes of protein decomposition, will often under similar conditions produce characteristic and pure colonies.

The organisms which are usually associated with *B. putrificus* in attempts at isolation are found to be spore formers, either aerobes or anaerobes. It is impossible, therefore, to eliminate them, by the heat method. Re-plating likewise proves unsuccessful. By replacing these associated organisms with known non-spore-bearing forms, however, a method was soon devised of isolating *B. putrificus*. *B. coli* was first employed, and after, definite periods of associated growth it was destroyed by heating the mixture at 80°C. for fifteen minutes.

Tubes of liquid agar were inoculated with putrefying material known to contain *B. putrificus*, and with varying amounts of *B. coli* cultures, and poured into Petri dishes. The plates were incubated in the Novy jar. Some of the colonies which developed were found, by microscopic examination, to be made up of *B. putrificus* and *B. coli*. Subculture from such colonies into eggmeat medium, and heating at 80°C., yielded pure cultures of the putrificus bacillus.

The following modification of the above method of isolating obligate anaerobes has greatly simplified it without lessening its efficiency. By substituting *Staphylococcus aureus* for *B. coli*, symbiotic growths (mixed colonies) were readily obtained by the usual method of open or aerobic culture. The amounts of the putrefying material and of the staphylococcus suspension were varied in order to obtain the most favorable balance of the two in the culture plates. After incubation at 37° for three or four days some of the irregularly-shaped yellow colonies were subcultured in egg-meat medium, in which putrefaction and protein liquefaction occurred after incubation for a few days at 37°C. Subsequent heating at 80° destroyed the staphylococcus, leaving the putrificus in pure form.

Five isolations were effected by the above improved method.

A SECOND METHOD

In a small number of our putrefying mixtures *B. putrificus* appeared to preponderate over the other organisms. This preponderance in numbers was easily observed in stained prepara-

tions. For such bacterial mixtures the following plan was evolved.

A loopful of the material is suspended in sterile water and well shaken. A direct count is then made, of the morphologically characteristic putrificus spores per cubic centimeter of the diluted medium, irrespective of all other forms. The bacterial suspension is further diluted with water until it contains from 10 to 50 of these spores to the cubic centimeter. Amounts of this diluted material varying from one loopful to 0.1 cc. are introduced into a series of egg-meat medium tubes. After inoculation with *Staphylococcus* the egg-meat tubes are incubated at 37°C. for a week. Of these a few will as a rule contain only *B. putrificus* and the *Staphylococcus*, which upon heating will yield pure cultures of the anaerobe in question. It is to be noted that this procedure is especially applicable when the original material contains a relatively large number of *B. putrificus* spores.

Three isolations were effected by this method.

CULTIVATION OF OBLIGATE ANAEROBES

As has been intimated, a very satisfactory way of cultivating anaerobes after they are once isolated is by symbiosis with some aerobic non-sporing organism under so-called aerobic conditions, or more correctly, by the usual aerobic methods. All of the aerobes employed by us proved equally effective in this connection. Tubes of egg-meat medium thus inoculated (*B. putrificus* and an aerobe) undergo vigorous putrefaction in the course of a few days, usually within three or four. Whenever the anaerobe is desired in pure culture heating at 80°C. readily removes the associated organisms. The most satisfactory medium for growing and maintaining putrefying organisms was found to be the egg-meat medium previously described by Rettger (1906). In this medium the putrefying anaerobes develop very rapidly and their putrefactive properties are well displayed. They remain viable for practically indefinite periods of time. Old tubes which had been kept in this laboratory for

at least two years readily yielded characteristic growths on transplantation to a new medium.

All of the strains of *B. putrificus* isolated by us exhibit a peculiar reluctance in undergoing development and in attacking the egg-meat medium in pure culture. The putrefaction is very much delayed, and does not begin as a rule until twenty to thirty days after the beginning of incubation under anaerobic conditions. When once begun, however, the putrefaction is rapid and typical. This is in striking contrast with the other well-known putrefyers (*B. oedematis* and *B. chauvei*) which usually begin to decompose the protein within a period of three or four days. This delayed putrefaction of *B. putrificus* occurs only in pure cultures. When growing in symbiosis with another organism, preferably an aerobe, it very quickly assumes a putrefactive rôle. The nature of this symbiosis is now under investigation. It will be of special interest to determine whether the associated organism favors putrefaction merely by rendering the conditions strictly anaerobic, or whether there are intermediary products of metabolism of the aerobes which favor the development of the obligate anaerobe.

It is quite apparent from a review of the literature that previous investigators have not been working with pure cultures of *B. putrificus*, as this phenomenon of delayed putrefaction which appears to be a constant feature of pure cultures of the organism has not been pointed out by them. We have attempted at different times to obtain pure cultures from other laboratories. The few cultures which were acquired in this way were found to contain other organisms.

The possible application of these methods to the isolation and cultivation of other fastidious anaerobes, particularly *B. tetani* and *B. welchii*, presents an interesting subject for investigation. Other media would be required, however, in place of the egg-meat mixture. Experience with the tetanus bacillus has shown how difficult it is to obtain this organism in pure culture, and it is not improbable that many of the so-called pure cultures of *B. tetani* are mixtures of this anaerobe and some other organism which is highly favorable to the growth and perhaps even to the toxin production of the tetanus bacillus.

REFERENCES

BIENSTOCK 1899 Untersuchungen ueber die Aetiologie der Eiweissfäulniss. Arch. f. Hyg., **36**, 335–389.

BIENSTOCK 1901 Milchfäulniss, Verhinderung der Fäulniss durch Milch. Arch. f. Hyg., **39**, 390–427.

RETTGER, L. F. 1906 Studies on putrefaction. Jour. Biol. Chem., **2**, 71–86.

RETTGER, L. F. 1906 Further studies on putrefaction. Jour. Biol. Chem., **4**, 45–55.

A PRELIMINARY REPORT UPON SOME HALOPHILIC BACTERIA

EDWIN LEFEVRE AND LESTER A. ROUND

Bureau of Chemistry, United States Department of Agriculture, Washington, D. C.

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While studying the microörganisms concerned in the fermentation of cucumber pickles a group of bacteria were isolated and studied which appear sufficiently interesting to deserve special comment. These organisms were isolated from the scum which grows on the surface of the brine after active fermentation (respiration) has ceased. The interesting fact about these organisms is that certain members of the group demand an appreciable concentration of sodium chloride for development.

HISTORICAL

Many investigators, among others Forster (1889), de Freytag (1890), Petri (1890) and Peuch (1887) have studied the salt tolerance of some of the well-known species of pathogenic bacteria such as *B. tuberculosis*, *B. typhosus*, *B. diphtheriae*, *Vibrio cholerae*, *B. anthracis*, and the bacillus of swine plague (Schweinrotlauf). Many of these types were found surprisingly resistant, being still living and virulent after months of exposure to saturated salt solutions. Sperlich (1912) studied the reaction of bacteria found in air, water and soil to varying percentages of salt. He found that many organisms grew better in the presence of a certain percentage of salt, the optimum varying from 0.5 per cent to 5 to 6 per cent of salt in the case of *Bact. constrictium* (Zimmermann).

Farlow (1867, 1886), Poulsen (1879–1880), Edington (1887), Leyet (1887), Ewart (1887), Le Dantec (1891), Hoye (1901, 1904, 1906, 1908), Beckwith (1911), Kellermann (1914–1915)

and Bitting (1911), have studied the bacteria concerned in the reddening of salted fish. The bacteriology of this phenomenon seems to have been fairly well worked out. Kellermann speaks of other non-pigment-forming bacteria associated with the causative agent but none of these investigators have called attention to a group of bacteria that demand for growth a certain percentage of sodium chloride. In this respect we believe the cucumber brine organisms to be unique.

Rapin and Grosseron (1914) have studied the possibilities of bacterial contamination of salt during the process of refining and Wolff (1914) made a study of highly refined salt used for butter making and of a less highly refined grade used for cheese making. Both investigators found that the more highly refined the salt the smaller the number of microörganisms present. Wolff showed that many of the bacteria found had a very marked effect upon butter when applied in pure culture to sterile material.

EXPERIMENTAL

From the pickle scum mentioned above 21 bacilli were isolated. The brine from which the scum was taken contained approximately 10 per cent of salt. The medium used for isolation was cucumber juice agar to which 10 per cent of sodium chloride had been added. On this medium at room temperature there appeared first a large number of rugose, cretaceous, raised colonies of mycoderma, in appearance quite characteristic of the scum as it appears on the surface of the pickle vats. After a few days smaller, creamy, glistening, colonies appeared, 1 to 2 mm. in diameter. These colonies were raised, opaque, with entire edge and of a butyrous consistency. Many of the colonies when viewed from the bottom of the plate had a very bright red center with a dark green periphery. When transferred to cucumber agar slants with ten per cent salt no further chromogenic action was noted except, in some cases, a browning of the medium underneath the stroke. It was found that these organisms would grow apparently just as well on meat extract medium with 5 per cent salt as on the cucumber medium and so for

further transfer of stock cultures and for differential purposes meat extract medium with 5 and 10 per cent salt was used. A study of the cultural characteristics of the organisms divided them into five groups as follows:

Group 1. Non-motile, non-spore-bearing bacilli, average size 0.5 by 2μ. On salt agar smooth, opaque, white, glistening colonies after forty-eight hours at 30°C.

Group 2. Non-motile, non-spore-bearing, Gram-negative bacilli 0.5 by 4 to 6μ. Abundant opaque, glistening colonies on salt agar. Motility doubtful.

Group 3. Gram-positive bacilli 0.5 by 4μ showing elliptical terminal spores. On salt agar plates there were formed at first large myceloid colonies which did not reappear in subcultures.

Group 4. Short, non-spore-forming, Gram-positive, average size 0.4 by 3μ with a tumbling form of motility. Colonies on salt agar convex, round, glistening, butyrous. No spores.

Group 5. Small, motile, non-spore-forming, Gram-positive bacilli 0.3 by 2μ. Colony formation not uniform.

Other cultural characteristics are shown in the following table.

The most conspicuous feature about these organisms is their ability to grow in highly concentrated salt solutions, the majority growing readily in a 25 per cent solution of sodium chloride.[1] In only one of the groups (no. 1), could growths be obtained without the aid of added salt. The optimum temperature was 30°C. But under the most favorable conditions growth was slow. The organisms were very sensitive to acids. Inoculated into pickle juice with varying degrees of acidity, all failed to grow above + 1. All except 3 cultures grew in salt broth with a P_H value of 6. In broth with a P_H value of 5, growth occurred in but one culture.[2]

All the cultures were facultative, although growth was more abundant in the presence of air.

No indol was formed and nitrates were reduced by but one of the groups (group 5).

[1] Medium containing 25 grams of sodium chloride in 100 cc. This method was followed in making all the media containing salt.

[2] The Colorimetric Determinations of Hydrogen Ion Concentration and Its Application in Bacteriology by Clark and Lubs, Journ. Bact., **2**, nos. 1, 2 and 3.

Table showing cultural characteristics of five groups of Halophilic organisms

GROUP NUMBER	GRAM STAIN	LIQUEFACTION OF GELATIN	ACTION IN MILK	GROWTH ON POTATO	INDOL PRODUCTION	NITRATE PRODUCTION	ACID PRODUCTION* IN			SPECIAL CHARACTERISTICS	RELATION TO SALT	
							Glucose	Lactose	Sucrose		Minimum requirement	Maximum requirement
											per cent	*per cent*
1	0	0	No change in plain milk after 20 days but milk with salt added coagulated in 18 days	Moderate raised, glistening	0	0	0	0	0	Decided ropiness in sugar broths	0.0†	15
2	0	0	Coagulated in 5 to 10 days	Raised, spreading, glistening	0	0	0	0	0	Odor of butyric acid in sugar media	5	25
3	+	+	Coagulated in 5 days	Moderate, raised, spreading	0	0	+	0	+	Spore formation	5	25
4	+	0	Not coagulated	Membranous, flat, glistening	0	0	+	0	0	Characteristic growth on potato resembling a coating of shellac	5	25
5	0	0	Not coagulated	Moderate, smooth and glistening	0	+	+	0	0	Cultural characteristics not uniform as in other groups	5	25

* If medium titrated less than 1 per cent normal acid to phenolphthalein, it was recorded as a negative fermentation.

† Ordinary nutrient broth containing 0.5 per cent sodium chloride.

These organisms show great resistance to drying. Thoroughly dried out cultures kept in the laboratory for one year were easily revived by adding salt broth to the tubes.

The form of growth shown in salt broth by most of these cultures was quite characteristic. In this medium there was slowly formed a moderate, uniform cloudiness with a light flaky pellicle, which later became thick and creamy. After several days' incubation, the pellicle broke up, forming viscid, stringy material suspended horizontally in the medium. This settled later and formed a viscid mass at the bottom. In group 1 marked ropiness of the liquid media occurred.

While the tolerance of certain organisms for high concentrations of sodium chloride and the favorable influence of certain concentrations upon growth has been determined for many organisms, no one, so far as the writers are aware, has called attention to a group that absolutely demand sodium chloride for their development.

Investigations have indicated that the chlorides are not essential to the growth and development of bacteria and experience has shown that the presence of salt in more than small percentages is inhibitory to the growth of many types (Sperlich, 1912). All these organisms showed a marked preference for salt and all except group 1 apparently demanded a certain concentration, 2 per cent, for their growth.

No explanation of this phenomenon is offered but the inquiry suggests itself as to whether they are essentially salt organisms which by long environmental influences have come to require salt in high concentration as essential to their vital processes, such as we might expect to find in sea water, or whether they are only common saprophytic bacteria which by growth for only a short time in salt solution have developed mutants which demand salt for their growth.

The question of the influence of osmotic pressure upon these organisms has not been studied. There is the possibility that the essential factor is not sodium chloride but a certain osmotic pressure which is necessary to create a suitable environment for growth. If it is only an increase in osmotic pressure that is

demanded, other salts in proper concentration would act in the same manner as sodium chloride. Further work on this point is contemplated.

REFERENCES

BECKWITH 1911 Centralbl. f. Bakt., II Abt., **60**, 351–354.

BITTING Bull. Bur. of Chem., U. S. Dept. Agric., 133. (1911).

EDINGTON 1887 Sixth Ann. Rep. to the Fishery Bd. for Scotland, 207–214.

EWART 1887 Sixth Ann. Rep. to the Fishery Bd. for Scotland, 204–207.

FARLOW 1867, 1886 Rep. U. S. Comm. of Fish and Fisheries, 1867, 969–979. Vegetable parasites of codfish (Bull. U. S. Fish Comm. 1886, **6**, 1-4).

FORSTER Munch. med. Wchnschr., 1889, No. 29, 497.

FREYTAG 1890 Arch. f. Hyg., **11**, 596.

HOYE 1901, 1904, 1906, 1908 Bergens Museums Aarbog 1901, No. 7, 2 Hefte; 1904, No. 9, 3 Hefte; 1906, No. 12, 2 Hefte; 1908, No. 4, 1 Hefte.

KELLERMANN 1914–1915 Centralbl. f. Bakt., II Abt., **42**, 398–402.

LE DANTEC 1891 Ann. de l'Inst. Past. I.S. 656, 667; Comp. Rend. de soc. de biol. 1906, 61, 136–138; Abs. Centralbl. f. Bakt., II Abt., **19**, 326, 1907.

LEYET 1887 Bull. U. S. Fish Commission, **7**, 90–95.

PETRI 1890 Arb. a.d. Kaiserl. Gesundheits Amte, **6**, 292. Abs. in Centralbl. f. Bakt., 1890, **8**, 596.

PEUCH 1887 Comp. Rend., **105**, 285.

POULSEN 1879–1880 Vidensk Meddel fra d. naturh. i Kjobenhaven f. 1879–1880. 231–254.

RAPIN AND GROSSERON 1914 Cited by Wolff, Milchwirtsch. Centralbl., **43**, 544.

SPERLICH 1912 Centralbl. f. Bakt., II Abt., 406.

WOLFF 1914 Centralbl., **43**, 544.

YEAST AUTOLYSATE AS A CULTURE MEDIUM FOR BACTERIA

I. J. KLIGLER

From the Laboratories of the Rockefeller Institute for Medical Research

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The necessity for conserving meat and meat products during the war has rendered a search for cheaper sources of nutritive media for bacteria highly desirable. Douglas and Gordon in England, and more recently Meyer in this country, have proposed the use of peptic and tryptic digests of animal tissues as a substitute for meat extracts and peptones. These materials cheapen the cost of media but necessitate the use of a digestive enzyme, usually varying in potency, and of considerable amounts of valuable animal protein. It seemed that some non-animal protein might prove equally satisfactory.

Yeast naturally suggested itself as a possible substitute. Extracts of yeast have been used in the past to reinforce peptone culture media. Furthermore, yeast readily undergoes autolysis, in the process of which there are formed varying proportions of so-called peptones and aminoacids, while the vitamines remain intact. Thus yeast autolysate may be expected to contain all the nitrogenous elements required for the growth of bacteria. However, the proportions of the individual nitrogenous elements vary with the conditions and duration of the autolysis. The extent of the influence which such variations may have on the growth of bacteria cannot be predicted on theoretical grounds. Hence it became necessary to search empirically for the conditions required to obtain from yeast a satisfactory medium for bacteria.

METHOD OF PREPARATION

In addition to its cheapness the yeast medium is, if anything, easier to prepare than meat extract or meat infusion media.

NUMBER OF EXPERIMENTS	COMPOSITION OF MIXTURE	INITIAL P_H	TESTS, NUMBER OF	TIME OF INCUBATION	P_H	CHEMICAL TESTS				BACTERIOLOGICAL TESTS		
						Nitrogen content per 100 cc.		P_2O_5 per 100 cc.	Ash per 100 cc.	Broth		
						Total	Amino			Str.	Pneu.	Mycobacterium phlei
				days		grams	grams	grams	grams			
1	200 cc. crude brewers yeast to 1000 cc. distilled water, 30 cc. M/15 NaH_2PO_4, 10 cc. chloroform; incubated at 37°C.	5.0	1	2	6.1	0.0818	0.0429			+++	++	++
			2	4		0.1128	0.0617			++	+	++
			3	5	6.1	0.0991	0.0566			+++	+	++
2	Same mixture	6.0	1	2	6.5	0.0930	0.0492			+++	++	++
			2	4		0.1184	0.0662			+++		++
			3	5	6.5	0.1103	0.0657			+	++	++
3	Same mixture	7.0	1	2	7.3	0.0846	0.0455			++	+++	++
			2	4		0.1243	0.0665			+++	+++	++
			3	5	7.6	0.1159	0.0640			+++	+++	++
4	400 cc. crude brewers yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4, 10 cc. chloroform, incubated at 37°C.	6.1	1	4	6.1	0.348	0.1803			+++	+	++
5	Same mixture	6.5	1	4	6.5	0.379	0.2078			++	−	++
6	150 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4, 5 cc. chloroform; incubated at 37°C.	6.1	1	3	6.1	0.2520	0.1760	0.1176	0.384	++	+	++
7	200 grams drained yeast, rest same as no. 6	6.1	1	3	6.1	0.2892	0.1922	0.1688	0.485	+++	++	++
8	200 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4, 5 cc. chloroform	6.1	1	2½	6.1	0.2603	0.1314			+++	++	++
9	400 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4; adjusted to P_H and autoclaved	7.0	1			0.128	0 0340			−	−	++
10	Nos. 8 and 9 mixed before broth was prepared	10				0.2342	0.0898					
11	Broth, Fairchild peptone					0.3663	0.0522			+++	+++	++
12	Veal broth, Witte peptone (Meyer and Stickel)					0.3472	0.0359	0.2	0.4088			

BACTERIOLOGICAL TESTS						TOXIN	
Broth							
Typho-sus	Dys. F.	Dys. Sh.	Diph.	Staph.	Indol B.Coli	B. diphtheriae	B. dysenteriae
++	+++	+++	+++	+++	+		
++	+++	+++	+	+++	++		
++	+++	+++	++	+++	++		
++	+++	+++	+	++	+	Not tested	Not tested
++	+++	+++	++	+++	+		
++	+++	+++	++	+++	+		
++	+++	++	++	+++	+++		
++	+++	+++	++	+++	+++		
++	+++	+++	++	+++	+++		
++	+++	+++	++	+++	+++	Filtrate 10 day broth culture inoculated intraperitoneally into guinea pigs (1) 0.3 cc., no effect (2) 0.6 cc., died 3d day (3) 1.0 cc., died 2nd day	2cc. filtrate 4 day broth culture in ear vein of rabbit; paralysis 48 hours. Died in seventy-two hours.
++	+++	+++	+	+++	+++	Same as above (1) 0.3—no effect (2) 0.6—no effect (3) 1.0—no effect	Same amount as above; no effect.
++	+++	+++	++	+++	+++	1.0 cc. filtrate of 10 day culture inoculated into each of 2 pigs. Recovered	
++	+++	+++	++	+++	+++	Same as above 1 died in 2 days 2 died in 4 days	
++	+++	+++	++	+++			Not tested
++	+++	+++	−	+++			
+++	+++	+++	+++	+++	+++	+	+

Two hundred grams of drained or centrifuged brewer's yeast are suspended in a liter of water, 2 grams of NaH_2PO_4 are added as buffer and the reaction adjusted by the addition of N/NaOH, to P_H6.1. Then 5 cc. chloroform are added and the mixture is thoroughly shaken and incubated at 37°C. for two days. It is necessary to shake the flask occasionally during the incubation, to maintain sterility.

At the end of the incubation period the reaction is brought to P_H7.4 and the autolysate heated in a water bath or in the Arnold for thirty minutes. It is then filtered through paper, tubed and autoclaved.

Agar is prepared by adding 15 grams of agar directly to 1 liter of the unfiltered autolysate, and stirring thoroughly to immerse and soften the agar shreds or powder. The mixture is then heated on the free flame or in the autoclave until the agar is completely dissolved. The reaction is then adjusted to P_H7.4, the mixture is heated on a water bath or in an Arnold for half an hour and the partially clear supernatant fluid is decanted to an Erlenmeyer flask or other vessel. The agar is then cooled and whole egg added to clear. The medium is finally steamed in the Arnold for one half to three quarters of an hour, filtered, tubed and autoclaved.

Special media may be prepared with yeast broth or yeast agar as a base according to the usual procedure.

RESULTS

Table 1 contains a summary of the experimental data on which this report is based. About 300 cc. of crude brewer's yeast or 200 grams of the drained material are sufficient to make 1 liter of broth, the total nitrogen and ash of which are approximately equal to, while the amino N is approximately four to five times as high as that of ordinary broth. The hydrogen concentration at which autolysis is allowed to proceed is an important factor in determining the end products and the suitability of the filtrate for culture media. The optimum concentration is P_H6.1.

Brewer's yeast was used in all the experiments reported in this paper. Both the crude material and the drained and centrifuged yeast were tested. Comparative tests were also made to determine the effect of heat or filtration on the efficacy of the medium. Chemical analyses of the total nitrogen and amino acid content of the autolysate media were made to parallel the cultural tests. The presence of tryptophane was determined by the ability of *B. coli* to produce indol. Toxin production in the case of *B. dysenteriae* and *B. diphtheriae* was tested by animal inoculation.

As a result of these experiments it may be concluded, tentatively at least, that yeast autolysate furnishes a suitable medium for the cultivation of all the common bacteria. The various members of the colon typhoid group thrive better in this medium than in ordinary broth. *B. coli* produces an abundance of indol in twenty-four to forty-eight hours. The Shiga type of *B. dysenteriae* on incubation of three to four days at 37°C. yields a toxin which produces the typical reaction in rabbits—paralysis after forty-eight hours, with typical intestinal lesions. The streptococcus and staphylococcus grow readily, but the pneumococcus does not thrive on the yeast autolysate media. The *B. diphtheriae* grows as well on these media as on ordinary agar or in broth. In the yeast broth it produces toxin, but apparently not of as high a potency as in some peptone broths.

It is not possible at present to predict the extent of the usefulness of yeast autolysate in culture media. Preliminary tests indicate that it can be used as a base for the various special media. Endo and brilliant green media prepared with yeast agar give entirely satisfactory differentiation.

SUMMARY AND CONCLUSIONS

Experiments are presented indicating that yeast permitted to undergo autolysis may serve as a cheap substitute for more expensive animal proteins or their digestion products. The autolysate contains a high percentage of amino nitrogen and a relatively small amount of the higher nitrogen complexes. The

fact that some bacteria, notably pneumococcus, meningococcus, etc., do not thrive as well in the yeast broth as they do in beef infusion media, would indicate that the higher nitrogen complexes—polypeptides, etc.—play some part in bacterial nutrition. On the whole, however, it seems that the yeast autolysate media are entirely satisfactory for the cultivation of the less delicate pathogenic and saprophytic bacteria. Endo and brilliant green plates made with this medium give entirely satisfactory results.